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13. ABSTRACT (Maximum 200 Words)

PSMA is a membrane protein that is highly expressed in the prostate and prostate cancers. It is also strongly expressed in the neovasculature of most solid tumors. PSMA is therefore an excellent target for therapy. We have examined the biology of PSMA relative to the possibility of it serving as a target for prodrug or targeted small molecule therapeutics. We observe that although PSMA can hydrolyze glutamate from polygammglutamated methotrexate, that not all antifolate serve as substrates that can have their antitumor activity enhanced by PSMA. We have also observed that other forms of PSMA such as PSM' and PSMA-like proteins, lack folate hydrolase activity and thus will not interfere with the prodrug activities of PSMA. Ligands that bind with high affinity to PSMA may have activity as imaging agents, but we have not observed that ligands increase the rate of internalization of PSMA and thus targeted toxins need to be designed that do not need to be internalized for their antitumor activity to be expressed.

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Final Report for DAMD17-00-1-0043: Development of a Biological Basis for PSMA Targeting in Prostate Cancer. P.I.: W.D.W. Heston Ph.D.

Introduction:
PSMA is a strongly expressed protein in prostate cancer. It is a type two membrane protein with a short 19 amino terminal protein inside the cell, a membrane spanning region from amino acids 20 to 43, and the rest of the 750 amino acids outside of the cell. We discovered that PSMA has unique hydrolytic activity as a carboxypeptidase in releasing glutamate either from polygammaglutamated folate as a folate hydrolase or from N-acetylaspartylglutamate, NAAG, as a neuropeptidase. We observed that cells expressing PSMA were killed by polygammglutamated methotrexate while cells not expressing PSMA were not killed which led us to hypothesize that glutamated prodrugs that were substrates for PSMA may serve as therapeutic agents in the treatment of prostate cancer and to understand the biology of PSMA function served as the basis of our application for funding.

Body:
Aim one of our proposal is focused on characterizing the structural, enzymatic, and transport activity of PSMA and PSMA like proteins for the rational development of biology based targeting strategies.

We proposed to clone enzymatically active PSMA and PSM' which could be used in x-ray diffraction: We have cloned enzymatically active PSMA. The cloned PSMA recombinant protein lacked the intracellular and transmembrane domains, but retained most of the extracellular domain and contained the folate hydrolase portion. The purpose of generating the recombinant PSMA protein was to focus on x-ray crystal diffraction. The PSM' protein that is the alternatively intracellular protein and is not glycosylated was found to lack enzymatic activity. Working with PSMA has been a major problem because the protein is extensively glycosylated and glycosylation interferes with x-ray diffraction structural determinations. To attempt to circumvent the problem, we have generated a number of mutants which have the glycosylation site mutated to see if we could identify a region in which we would have enzymatic activity without glycosidation. We have found that this is not possible. Even minor perturbations in glycosylation eliminate folate hydrolase activity. We have published a paper on this in the journal *Prostate*.

In terms of the biology of PSMA, a concern was the presence of PSM' an alternative spliced form of PSMA that would be intracellular and may compromise a prodrug strategy and PSMA-like gene which is expressed in non-prostate tissues and may compromise PSMA pro-drug targeting. The alternative spliced form of PSMA, PSM' is an alternative spliced form of PSMA which lacks the transmembrane domain and is cytosolic. We initially were concerned about its presence because we believed that it would have activity that could interfere with prodrug activity. To our surprise, we found that intracellular versions of PSMA lacked enzymatic activity. We published this result in the journal *Prostate*. In work with colleagues, we discovered that the monomer form of PSMA has no enzymatic activity. It is only the dimer form of PSMA that has activity.

This has major implications for PSMA functioning as a target enzyme for prodrug activation, because one would want the tumor cells to have PSMA in the dimer form during prodrug activation. PSMA will change from one form to the other without this requiring a covalent bond as would occur with sulhydryl's for instance. So this is a dynamic equilibrium. We don't as yet understand what induces the change in forms. So our future studies will focus on identifying methods to increase the enzymatic dimer form of PSMA in prostate tumor cells. This collaborative work was published in Proceedings of the National Academy of Sciences.

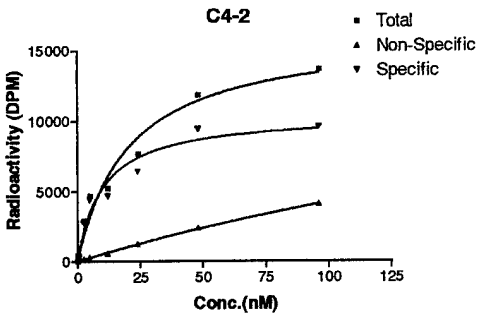
The other aspect is internalization. What is responsible for the internalization of the PSMA protein. We initially thought it was the dielucine motif of the short intracellular domain. In collaboration with colleagues, we determined that there is a unique motif, being MXXXL. This explains why the PSMA protein is found at the apical surface rather than the basolateral surface as would be predicted from the di-leucine motif. We continue to develop means to optimize protein internalization as well. This collaborative effort was published in Molecular Biology of the Cell.

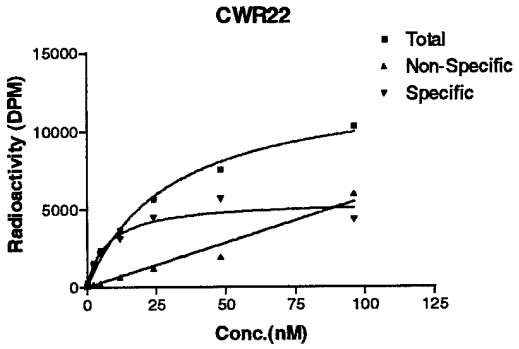
Aim two: In the second specific aim we were to test polygammglutamated derivatives for antitumor activity. We have been stuck by the specificity of the hydrolytic function of PSMA. It is more restricted in enzymatic hydrolysis than we first believed, so that not all gamma-glutamated antifolate derivatves are in fact substrates.

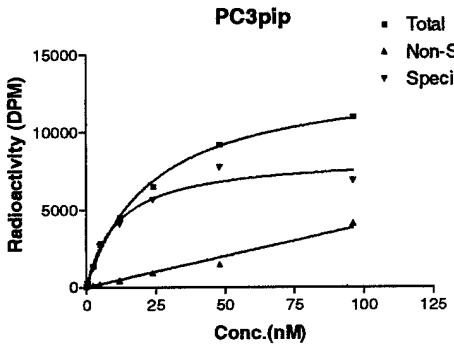
We have examined the binding of methotrexate polygammglutamates and a number of substrates, agonists and antagonists, and they all confirm that there is almost no binding to the monomer form, that they are binding in fact binding the dimer form.

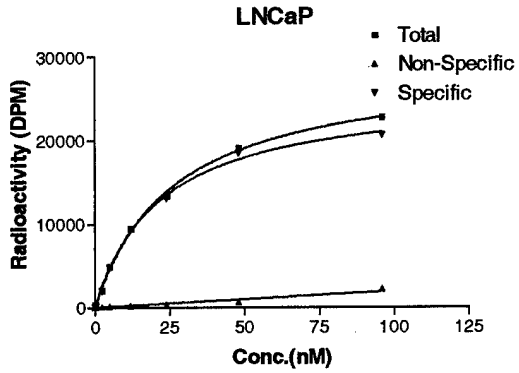
We have tested methotrexate polygammgluatmate in vivo in animal models of PSMA expressing tumor cells and have observed growth inhibition and have much less toxicity of methotrexate polygammaglutamate when compared with methotrexate. We have observed growth suppression, but after three weeks of constant exposure, the animals began to develop toxicity to the methotrexatepolygammaglutamate. So we are contemplating means to circumvent this toxicity with further enhancement of tumor selective delivery.

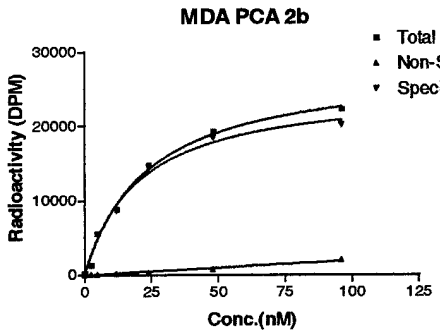
We were disappointed that none of the potential ligands of PSMA's hydrolytic enzymatic activity would induce PSMA to internalize. This decreased the potential of this aspect of ligand binding to become a potential means of drug delivery. However, we continue to pursue this aspect through new collaborations and are evaluating urea derivatives of glutamate to serve as toxin targeting vectors. We do find, as is shown in the figure, that ligands to the hydrolytic domain do demonstrate high affinity binding specifically to prostate cancer cells such as C4-2,Cwr22, LNCaP , MDA PCa-2b, and PC3pip cells, but not to cells such as PC-3, DU-145 or other cells which do not express PSMA (PC-3 and Du-145 binding data not shown). These are relative binding plots of the amount of tritiated ZJ-24 that binds to different cells relative to non-specific binding.



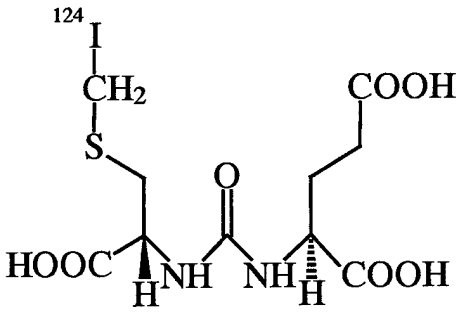


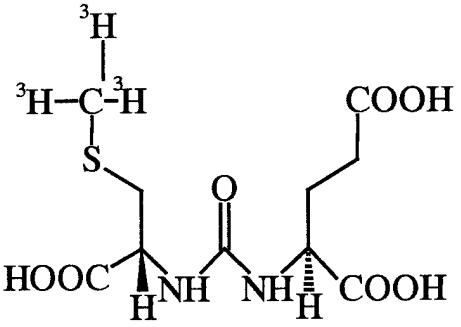






In the future, we are planning studies to determine whether positron emitting radionuclides of ZJ-24 might be able to serve as both an imaging agent as well as a therapeutic agent using I-124 modified ZJ-24. Even though we have found that these types of ligands do not induce internalization, we will determine whether toxins linked to these ligands will target the toxin to PSMA expressing cells.





Key research accomplishments:

We have identified a recombinant form of PSMA that has allowed us to discover that PSMA is able to undergo spontaneous inter-conversion between a monomeric and a dimeric form that does not require a covalent modification, and that all of the enzymatic activity or binding of ligands or antagonists occur with the dimer form. We have discovered that modification of any site of glycosidation eliminated hydrolase activity. Thus it will be unlikely that X-ray diffraction studies of the enzymatic active site of PSMA will be accomplished with current state-of the art techniques.

We have also determined that PSM' and PSMA-like genes are unlikely to interfere with pro-drug strategies aimed at PSMA, because neither is a membrane protein, and both are less likely to be extensively modified by glycosidation, and neither have been found to be enzymatically active.

We have also observed that methotrexate polygammglutamate is able to inhibit the growth of a PSMA expressing tumor in vivo, but that there is still toxicity associated with the polygammglutamate, even though it is substantially less than free methotrexate, and further targeting of the prodrug need to further increase the specificity of prodrug delivery. Further demonstration that each compound would have to be independently verified is based on our observation that folate antagonist Ly231514 even polygammglutamated was not more active on cells that expressed PSMA compared with those that did not.

We have evidence that suggests that because of the availability of high affinity ligands for PSMA that radioactive nuclides such a positron emitting nuclides may provide for imaging agents for PSMA and may also provide for therapeutic targeting depending on the radionuclide used.

Reportable outcomes since the previous report:

Ghosh, A., and Heston WDW: Effect of carbohydrate moieties on the folate hydrolase activity of the prostate-specific membrane antigen. Prostate 57:140-151, 2003.

Ghosh A., and Heston WDW: Tumor target prostate-specific membrane antigen (PSMA) and its regulation in prostate cancer. J. Cell Biochem. 91: 528-539, 2004.

O'Keefe DS, Bacich DJ, Heston WDW: Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate specific membrane antigen-like gene. Prostate 58: 200-210, 2004.

Collaborative Publications relevant to this program's specific aims:

Rajaskaran SA, Anilkumar G, Oshima E, Bowie JU, Liu H, Heston WDW, Bander NH, Rajaskaran AK: A novel cytoplasmic tail MXXXL motif mediates the internalization of prostate specific membrane antigen. Mol Biol Cell 14: 4835-4845, 2003.

Schulke N, Varlamova OA, Donovan GP, Ma D, Gardner JP, Morrissey DM, Arrigale RR, Zhan C, Chodera AJ, Surowitz KG, Maddon PJ, Heston WDW, Olson WC: The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy. Proc Nat'l Acad Sci., 100: 12590-12595, 2003.

Prior reports: 2001

Uchida A., O'Keefe DS., Bacich DJ., Watt F., Molloy PL., Heston WDW.: Prostate-specific suicide gene therapy using the newly discovered prostate-specific membrane antigen (PSMA) enhancer. Proc. Amer. Assoc. Cancer Res. 42:691, 2001. (Abst#3717)

Grasso YZ., Heston WDW.: Prostate-specific membrane antigen as a target for prodrug therapy: relationship between its folate hydrolase and endocytosis activities. Proc. Amer. Assoc. Cancer Res. 42:777, 2001. (Abst# 4169)

O'Keefe DS, Uchida A, Bacich DJ, Watt FB, Martorana A, Molloy PL, Heston WDW.: Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. Prostate 45: 1490157, 2000.

Prior reports 2002:

Schulke N., Donovan GP., Morrissey DM., Arrigale R., Varlamova T., Scalzo M., Israel RJ., Heston WDW.: Human prostate specific membrane antigen (PSMA) is naturally expressed as a noncovalent dimer . Proc AACR. New Discoveries in Prostate Cancer and Treatment. Dec 5, 2001.

O'Keefe DS., Ucida A., Bacich DJ., Milbank A., Heston WDW.: In vivo suicide gene therapy using the prostate-specific membrane antigen promoter enhancer. Proc AACR, New Discoveries in Prostate Cancer and Treatment. Dec 5, 2001

Oshima E., Rajasekaran A., Kim G., Ru N., Liu H., Heston WDW., Bander NH., Rajasekaran AY.: A cytoplasmic tail di-leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen. Proc AACR. New Discoveries in Prostate Cancer and Treatment. Dec 5, 2001.

Uchida A., O'Keefe DS., Bacich DJ., Watt F., Molloy PL., Heston WDW.: Prostate-specific suicide gene therapy using the newly discovered prostate-specific membrane antigen (PSMA) enhancer. Proc. Amer. Assoc. Cancer Res. 42:691, 2001. (Abst#3717)

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Personnel Involved. The following individuals have received salary support from the grant:

Warren D.W. Heston, Ph.D.
Dean J Bacich, Ph.D.
Ying Grasso, Ph.D.
Kelley Harsch, B.S.

Conclusions:
PSMA internalization requires a new internalization motif, MXXXL. This explains that the dileucine repeat is in fact not the motif functioning in PSMA's internalization and explains why PSMA is found at the cellular apical surface rather than the basal surface. We have identified that the addition of ligands to the hydrolytic domain of PSMA do not induce the internalization of PSMA and thus are not likely to be as active relative to antibodies which do induce internalization of targeted toxins. This suggests that low molecular weight approaches the need to develop toxins that can be active at the cell surface. We have identified that high affinity urea derivatives of glutamate have binding properties that would allow them to be developed as imaging agents and as vectors for therapeutic targeting.

We have identified that PSM' and other cytosolic versions of PSMA, are not enzymatically active and are unlikely to interfere with the pro-drug activation of PSMA. We have identified that even minimal changes in the glycosylation of PSMA eliminates enzymatic activity and thus reduces the likelihood that we will be able to develop a mutant that we will be able to determine the structure by X-ray diffraction studies.

References:

Ghosh A., and Heston WDW: Tumor target prostate-specific membrane antigen (PSMA) and its regulation in prostate cancer. J. Cell Biochem. 91: 528-539, 2004.

Appendices:

Ghosh, A., and Heston WDW: Effect of carbohydrate moieties on the folate hydrolase activity of the prostate-specific membrane antigen. Prostate 57:140-151, 2003.

Ghosh A., and Heston WDW: Tumor target prostate-specific membrane antigen (PSMA) and its regulation in prostate cancer. J. Cell Biochem. 91: 528-539, 2004.

O'Keefe DS, Bacich DJ, Heston WDW: Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate specific membrane antigen-like gene. Prostate 58: 200-210, 2004.

The Prostate 57:140 – 151 (2003)

Effect of Carbohydrate Moieties on the Folate Hydrolysis Activity of the Prostate Specific Membrane Antigen

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BACKGROUND. Prostate specific membrane antigen or PSMA has been recognized as one of the important cellular markers for prostate cancer, the expression of which is enhanced many fold in prostate cancer and other tumor neovasculature. PSMA is a type II membrane glycoprotein with a short cytoplasmic N-terminal region, a transmembrane domain, and a 701 amino acid extracellular portion with 10 potential N-linked glycosylation sites. PSMA is a folate hydrolase, which cleaves terminal glutamates from poly- and gamma-glutamated folates; and NAALADase, which hydrolyses alpha-glutamate-linked dipeptide, *N*-acetyl-aspartyl-glutamate (NAAG) and is a glutamate carboxypeptidase.

METHODS. In our study we have used various enzymes or site directed mutagenesis to remove sugar molecules from PSMA protein and studied its folate hydrolase function. We have performed a biochemical characterization of N-linked glycosylation of the various mutant proteins.

RESULTS. PSMA protein expressed in different prostate cancer cell lines is differentially glycosylated. Removal of sugar residues either enzymatically or by mutagenesis abolishes the enzyme activity of PSMA protein completely.

CONCLUSION. N-linked carbohydrate structures are important for the folate hydrolase function of the protein. Removal of sugars partially or completely causes PSMA to be enzymatically inactive, improperly folded, resulting in increased rate of degradation. *Prostate 57: 140–151, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **carbohydrates; N-linked glycosylation; folate hydrolase; PSMA; prostate cancer**

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men [1]. Tissue specific proteins serve as biomarkers of prostate cancer, and have been used in screening, diagnosis, and predicting disease progression. Prostate specific membrane antigen (PSMA), which was originally identified in LNCaP cells by its immunoreactivity with mAb 7E11-C5 is a membrane bound protein and has been designated as a target of imaging for prostate cancer [2]. PSMA is expressed in normal prostate [2–4] as well as nearly 100% of prostate carcinomas [5]. PSMA expression increased in higher-grade cancer, metastatic disease, and hormone refractory prostate carcinoma [4,5]. It has also been used to identify circulating prostatic tumor cells. In addition to prostate cancer, PSMA protein has been found to be strongly expressed in the tumor vascular endothelium of large number of solid tumors [6–8], while being absent in the endothelium of the normal tissues. A gene

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for human PSMA (2.65 kB) has been cloned, sequenced, and mapped to chromosome 11p11.2 [9]. The DNA sequence reveals that the protein-coding region has 54% homology with human transferrin receptor (nucleotide region 1250–1700, located in the catalytic domain of the protein). The protein encoded by the PSMA gene is about 750 amino acids long, contains a short N-terminal cytoplasmic region of 19 amino acids, a 24 amino acid transmembrane domain and remaining 707 amino acid extracellular C-terminal fragment. PSMA has two important enzymatic properties: (a) folate hydrolase activity, which hydrolyzes the γ-linked terminal glutamates from poly-glutamated folates. (b) NAALADase or *N*-acetyl-aspartyl-L-glutamate carboxypeptidase activity, with hydrolysis of the terminal α-linked glutamate from important neuropeptide NAAG. PSMA shows homology to a family of membrane carboxypeptidases, M28 [12]. They contain 2 co-catalytic zinc metallopeptidase sites, wherein there are two side-by-side peptidase enzymatic "pockets" and two coordinated zinc ions. Prediction program [Predict protein (PHD) mail server utility] analysis of secondary structure predictions revealed six domains within PSMA protein [10]. Domain A is the N-terminal cytoplasmic portion (including binding site of the 7E11.C5 antibody as well as dileucine repeat (internalization motif) found in membrane protein), domain B is the hydrophobic, transmembrane segment of PSMA including amino acids 20–39, domains C and D represents amino acids 40–144 and 173–248. Domain E is the catalytic domain (amino acid 275–596), domain F is of unknown function includes amino acids 597–756. There are linker regions rich in proline and glycine present between C and D (145–172) and D and E (249–273). The catalytic domain E contains two predicted zinc-coordinating sites. Asp^{387}, Glu^{425}, and His^{533} coordinate the first zinc atom. His^{377}, Asp^{387}, and Asp^{453} coordinate the second zinc atom. The amino acid Asp^{387} coordinates both metal atoms, which are commonly found in cocatalytic peptidases. The remaining domain, F, has no known function, although in transferrin receptor it is the ligand-binding site. The protein has 10 potential N-linked glycosylation sites.

We have analyzed the role of sugar moieties on the enzyme activity of PSMA molecule. In this article, we address the impact of N-linked glycosylation on the protein activity and protein stability.

MATERIALS AND METHODS

Cell Lines and Reagents

PC3 cells (catalog number: CRl-1435), MDA PCa2b cells (catalog number: CRL-2422), CWR22Rv1 (catalog number: CRl-2505) were obtained from ATCC. LNCaP C4-2 cells were obtained from Urocor Inc.

PSMA cDNA was originally cloned in our laboratory [4]. The PSMA expressing construct, which has been used for this study is a pIRES—Neo construct of PSMA. Among the antibodies, J591 monoclonal antibody was obtained from Dr. Neil Bander, Cornell Weil College of Medicine, NY. The PM1T 485.5 monoclonal antibody was obtained from Hybritech Corp. ^{35}S Methionine was obtained from Amersham (#AG1094). Recombinant PSMA protein (human PSMA extracellular domain) was obtained from Progenics, Inc. (Tarrytown, NY).

Generation of Glycosylation Mutants by Site-Directed Mutagenesis

Mutation of amino acid glycosylation sites was accomplished by Stratagene's QuickChange™ Site-Directed Mutagenesis Kit. Briefly, 100 ng of PSMA encoding cDNA (PSMA-pIRES-Neo construct) was taken; the plasmid was denatured at 95°C for 1 min, the oligonucleotide primers containing the desired mutation were annealed to it. Using non-strand displacing action of *Pfu* Turbo DNA polymerase, the mutagenic primers were extended and incorporated (temperature cycling conditions: 95°C, 30 sec; 55°C, 1 min; 68°C, 12 min for 18 cycles). The nicked circular DNA, which was generated in this process, is non-methylated, containing the desired mutation. In the next step, the methylated, non-mutated parental template DNA was digested with Dpn1 at 37°C for 1 hr. The circular, nicked dsDNA was transformed into XL1-Blue supercompetent cells, which will repair the nicks in the mutated plasmid. The colonies, which appeared on the LB-amp plates were picked up, plasmid DNA was isolated and sequenced to check correct incorporation of the desired mutation. Following are the sequences of the primers used for making mutants. Primers were obtained from Invitrogen, Inc. N336A (Asparagine at 336 position has been changed to Alanine); 5′senseN336A: CCTGGCTTTACTGGAGCCTTCTCTACACAAAAATC; 3′senseN336A: GATTTTTGTGTAGAGAAGGCTCCAGTAAAGCCAGG; N459A (Asparagine at 459 position has been changed to Alanine); 5′senseN459A: CTCATCTATAGAAGGAGCCTACACTCTGAGAGTTG; 3′senseN459A: CAACTCTCAGAGTGTAGGCTCCTTCTATAGATGAG; N476A (Asparagine at 476 position has been changed to Alanine); 5′senseN476A: GTACAGCTTGGTACACGCCCTAACAAAAGAGCTG; 3′senseN476A: CAGCTCTTTTGTTAGGGCGTGTACCAAGCTGTAC; N638A (Asparagine at 638 position has been changed to Alanine); 5′senseN638A: CACTTTTTTCTGCAGTAAAGAATTTTACAGAAATTGCTTCC; 3′senseN638A: CCAAGCAATTTCTGTAAAATTCTTTACTGCAGAAAAAAGTG.

Preparation of Cell Membrane Prep

Different prostate cancer cell lines endogenously expressing PSMA or PC3 cells transfected with different cDNAs of interest were harvested in PBS buffer. The cells (1×10^8) were scraped off from the flasks into PBS and pelleted by centrifugation. The cell pellet was resuspended in 50 mM Tris buffer, pH 7.5. The pellet was homogenized manually with a Dounce Homogenizer and was centrifuged at 1000*g* at 4°C for 5 min. The supernatant was separated and ultracentrifuged at 70,000*g* for 35 min at 4°C. The supernatant was discarded and pellet was washed with 3 ml 20 mM Tris buffer, pH 7.5. The pellet was finally resuspended in 1.5 ml of 50 mM Tris-HCl, pH 7.5, 0.1% Triton ×100 and was homogenized for 30 strokes to break up the pellet. The pellet was transferred to 1.5 ml eppendorf tubes and was spun at 1000*g* for 10 min. The supernatant was collected as the source of triton extracted PSMA and stored at 4°C.

Endoglycosidase Digestion

For enzymatic analysis, 2 μg of membrane preparations from different cell lines or 0.0002 μg of recombinant PSMA was incubated with Endo H (6 mU/sample) or increasing concentrations of Endo F1 (16, 32, and 48 mU/sample). Different samples were incubated overnight at 37°C and folate hydrolase assay performed.

Protein analysis. Twenty micrograms of protein equivalent membrane preparations from different prostate carcinoma cells lines endogenously expressing PSMA protein or PC3 cells transiently transfected with cDNAs encoding WtPSMA or its various mutants were harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Triton ×100) and subjected to treatment with a panel of glycosidases. They are either buffer treated control WtPSMA or treated with Endoglycosidase H (6 mU/sample), PNGaseF (5 U/sample), glucosaminidase (8 mU/sample), β-(1–4)galactosidase (3 mU/sample), Endo-*O*-glycosidase (2.5 mU/sample), Sialidase A (5 mU/sample). The protein samples were treated with different glycosidases, overnight at 37°C, analyzed by SDS–PAGE, Western blotted onto PVDF membranes and detected with PM1T 485.5 antibody (primary), followed by goat-anti-mouse HRP (secondary) and detected by ECL. Different glycosylated and deglycosylated PSMA bands have been shown by arrows.

Estimation of Protein Concentration and Western Blot Analysis

The protein concentration of the whole cell extract or the membrane preparations was estimated by BCA assay. For Western blot analysis, about 50 μg of protein was analyzed by SDS–PAGE and Western transferred onto a PVDF Immobilon-P membrane (Millipore). The blots were incubated in blocking buffer (3% fatty acid free BSA and 5% goat serum) for 1 hr followed by incubation with J591 [8] in 3% fatty acid free BSA in Tris buffered saline containing 0.5% Tween-20 (TBST) at 1:1000 dilution. The blots were washed thrice in TBST for 5 min each followed by incubation with peroxidase conjugated-goat anti-mouse secondary antibody [immunopure goat anti-mouse IgG (H + L)] 1:5000 dilution in 3% BSA-TBST. The blot was developed with ECL Chemiluminescent detection reagent (Amersham Pharmacia Biotech).

Folate Hydrolase Activity Assay

Two micrograms of protein (in 50 mM Tris-HCL, pH 7.5, 0.5% Triton ×100) from membrane preparations were incubated in the presence of 50 μM 4-Amino-10 methylpteroyl-di-3-glutamic acid (MTXglu2) (Schircks Laboratories, Jona, Switzerland) 50 mM Tris-HCl, pH 7.5 for total volume of 100 μl for 1 hr at 37°C. Reactions were quenched with 100 μl of Na_2HPO_4 (0.5 M). The reaction mixture (50 μl) is separated by Reversed-Phase HPLC with PRISM RP (5 μM, 50 × 4.6 mm) (Thermo-Hypersil Keystone Scientific Operations, Bellefonte, PA) with a mobile phase of 85% 0.05 M KH_2PO_4, pH 7.0, and 15% methanol. The UV absorbance was detected at 313 nm. The peak area was calculated by ESA CoulArray Software (ESA, Inc., Chelmsford, MA) for $MTXglu_2$ and MTX. The Folate hydrolase activity is reported as the average of three reactions per sample and is expressed as (nmoles of glu released)/mg of protein/hr.

NAALADase Activity Assay

Ten micrograms of equivalent protein from PC3 cells transfected with cDNA encoding WtPSMA or its different mutants were taken for NAALADase assay. The samples were harvested in the lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100 and the protein was quantitated using BCA protein assay reagent (Pierce, Rockford, IL). A total of 10 μg equivalemt protein was incubated at 37°C for 1 hr in a total volume of lysis buffer containing 4 μM *N*-acetyl-L-aspartyl-L-(glutamate-3, -4 -^3H) (Perkin Elmer life sciences, Boston, MA). The reaction stopped by the addition of an equal volume of 0.5 M NA_2HPO_4. The liberated glutamate was separated using ion-exchange chromatography as previously described [11]. Briefly, half the reaction was layered over polyprep prefilled chromatography column packed with AG-1-X8 formate resin, 200–400 mesh (BioRad, Hercules, CA) that had been pre-wet with distilled

water. The column was washed with 2 ml of 1 N formic acid, and the free-glutamate eluted in 2.5 ml of 1 N formic acid followed by measurement using scintillation spectrometry.

Immunofluorescence Analysis by Confocal Microscopy

For immunofluorescence analysis, the PC3 cells were seeded onto glass coverslips at the density of 1×10^4 cells and were transiently transfected with cDNAs encoding WtPSMA or its various mutants. At 48 hr post transfection, the cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were washed with PBS twice. The cells were permeabilized with PBS containing 0.2% Triton X-100, for 10 min at room temperature and washed with PBS thrice. The coverslips were washed with PBS three times and blocked with 3% BSA, 5% goat serum in TBST (0.05 M Tris-HCl, pH 7.6; 0.138 M NaCl, KCl 0.0027 M), followed by incubation with primary antibody PM1T 485.5 in 3% BSA in TBST (Tris buffered saline with 0.05% Tween-20). The coverslips were visualized with secondary antibodies, biotinylated-goat-anti-mouse antibody, and streptavidine-FITC subsequently. The coverslips were mounted on vectashield and visualized under confocal microscope.

Pulse Labeling Analysis

Briefly, 10×10^6 PC3 cells were seeded onto different wells of 6 well-dish and transfected with cDNAs encoding WtPSMA or its different glycosylation mutants with lipofectamine 2000 reagent (Invitrogen, Inc.) At 48 hr post-transfection, the cells were starved with methionine minus RPMI-1640 medium (Sigma, R7513) for 30 min, followed by labeling with 50 μCi of ^{35}S methionine supplemented labeling media for 1 hr, followed by chasing with cold methionine supplemented media (with 2% dialyzed fetal calf serum) for different periods of time 0, 2, 4, 8, 24, 48, 72 hr, respectively. The cells were harvested at the end of different chasing period with RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). One hundred micrograms of equivalent protein from each time point was immunoprecipitated with PM1T485.5 antibody and analyzed by SDS–PAGE followed by Western blotting onto PVDF membrane (Millipore). The blots were developed with primary antibody, PM1T485.5 antibody (Hybritech) at 1:1000 dilution, followed by secondary antibody goat-anti-mouse HRP (1:1000) dilution and developed with ECL reagent (Amersham).

RESULTS

Significance of Carbohydrate Structures on the Enzymatic Activity of PSMA Protein

PSMA is a type II globular membrane glycoprotein and has 10 potential glycosylation sites (Fig. 1) and they are distributed among various domains. Asn^{51}, Asn^{76}, Asn^{121}, Asn^{140} are located in domain C; the fifth residue Asn^{153} is located in the linker region between domains C and D. The sixth residue Asn^{195} is located in domain D; 7th (Asn^{336}), 8th (Asn^{459}), and 9th (Asn^{476}) are located in the catalytic domain. The 10th sugar attachment site (Asn^{638}) is located in domain F. To test the effect of glycosylation on the enzyme activity, we have pursued a strategy to deglycosylate the protein by Endoglycosidase H and F1. These enzymes cleave between GlcNAc of N-linked chitobiose units of high-mannose and hybrid type of glycans. In addition, EndoF1 cannot efficiently hydrolyze fucose containing hybrid oligosaccharides [12]. Following the treatment we tested the folate hydrolase activity of the protein. We have found that the overnight digestion with Endo H removes the sugar residues from the protein with the result of PSMA becoming enzymatically inactive (Fig. 2B). The Western blot analysis of the overnight Endo H (6 mU/20 μg of protein) treated PSMA protein (membrane preparation of C4-2 cells endogenously expressing PSMA) showed a band with faster mobility compared to control (incubated with Endo H buffer) (Fig. 2A), whereas the Endo F1digestion did not remove the sugar residues either partially or completely (no change in the band mobility of PSMA, Fig. 2A) even with increasing enzyme concentrations (16, 32, and 48 mU, respectively) indicating that PSMA expressed in C4-2

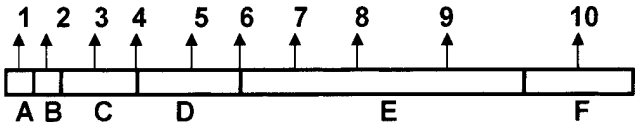

Fig. 1. Schematic diagram of PSMA protein showing different potential sugar attachment sites. PSMA is a globular protein with 10 potential glycosylation sites, which have been denoted here by arrows and numbers. Based on the model proposed by Rawlings and Barrett [10], the PSMA has a region of similarity to M28 peptidase family of metalloproteases and according to the prediction, there are six individual domains in the PSMA protein denoted by **A**, **B**, **C**, **D**, **E**, and **F**. Domain A is the intracellular cytoplasmic domain, domain B is the transmembrane domain, domains C, D, E, and F are extracellular domains. Domain E is the catalytic domain for coordinating Zn^{2+} ions. Different glycosylation sites are distributed across different domains of PSMA protein. They are as follows: 1: Asn^{51}, 2: Asn^{76}, 3: Asn^{121}, 4: Asn^{140}, 5: Asn^{153}, 6: Asn^{196}, 7: Asn^{336}, 8: Asn^{459}, 9: Asn^{476}, 10: Asn^{638}.

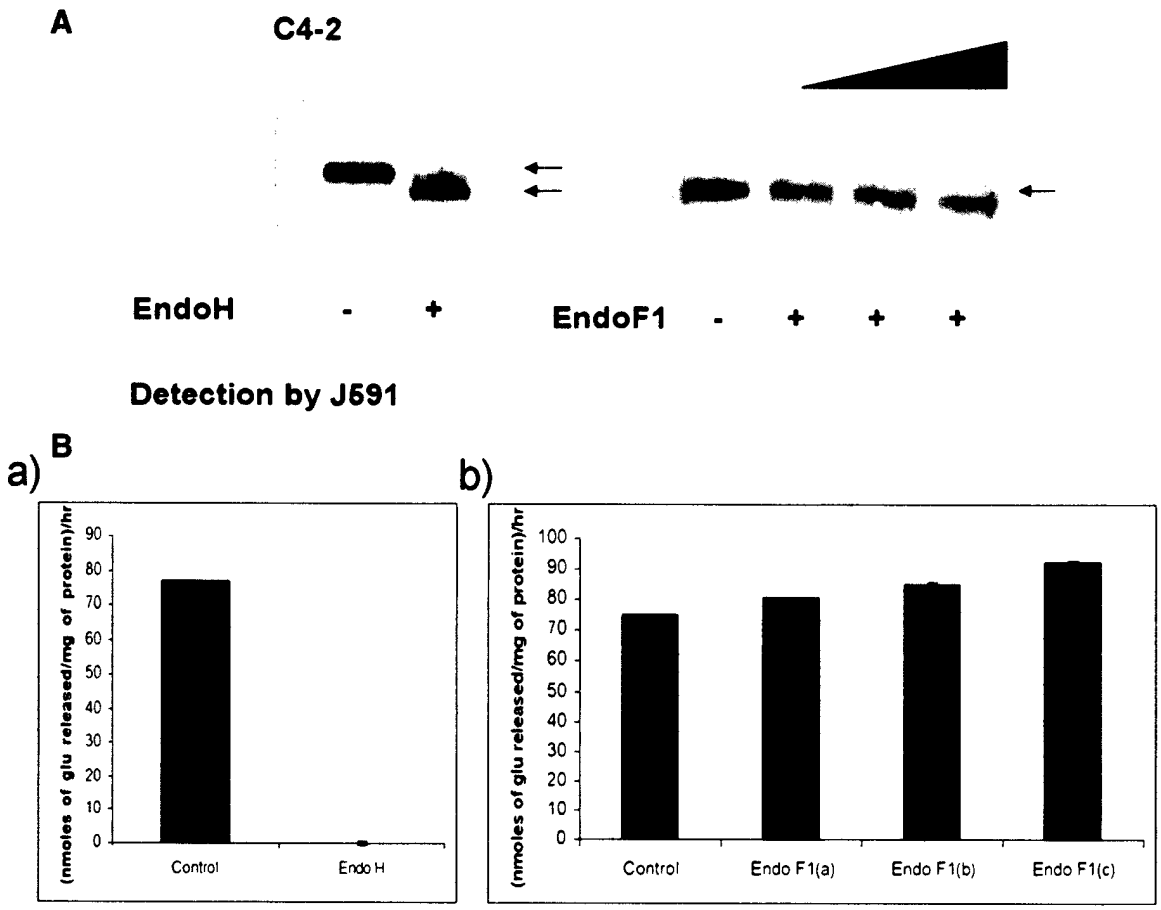

Fig. 2. Sugar residues are important for the enzymatic activity of the PSMA protein. **A**: Western analysis; (**B**) showing the folate hydrolase assay of different samples treated with Endo H and Endo FI (panels marked with Endo H and Endo FI, respectively) along with the buffer treated control. Twenty micrograms of membrane preparations of C4-2 cells were taken as the source of protein and treated with Endo H (2 mU/sample) and Endo FI (1), (2), and (3) are 16, 32, and 48 mU of enzyme/sample, respectively, overnight at 37°C. The proteins were analyzed by SDS–PAGE, Western blotted onto PVDF membrane and detected with J591 antibody. **B**: Folate Hydrolase assay: 2 μg protein equivalent membrane prep was used for folate hydrolase assay. The samples were separated on reverse phase HPLC and data have been plotted using ESA Coularray Software.

cells is composed of fucosylated oligosaccharides. Endo F1 treatment did not alter the folate hydrolase activity of the protein (Fig. 2B) significantly. This indicates that PSMA protein expressed in C4-2 cells is Endo H sensitive but Endo F1 resistant.

We have also tested the Endo H and Endo F1 cleavability of the recombinant PSMA protein expressed in Chinese hamster ovary cells (CHO) (Fig. 3). As we observed before, the recombinant PSMA protein was Endo H sensitive, but Endo F1 resistant (Fig. 3A). Endo H treatment reduces the folate hydrolase activity of the protein (Fig. 3B), but exhibits an increase in enzyme activity following treatment with increasing concentration of Endo F1 (16, 32, and 48 mU/0.0002 mg of protein). Treatment of protein with 48 mU of endo F1 showed an increase of about 25% of folate hydrolase activity compared to untreated control.

Role of Glycosylation Inhibitor on the Biosynthesis and Enzyme Activity of PSMA

To further establish that glycosylation is important for the enzymatic activity of the protein, we have expressed the protein in presence of specific glycosylation inhibitors. The PSMA protein encoding cDNA was transiently transfected into PC3 cells and synthesized in the presence of tunicamycin (10 μg/ml), which potently inhibits the first step in the N-glycosylation pathway. As seen in the Western blot (Fig. 4B, lane 2), the apparent molecular weight of PSMA expressed in the Tunicamycin treated PC3 cells was lower than that of the protein produced without the inhibitor. The various deglycosylated forms produced by the tunicamycin treated PC3 cells showed that the N-glycosylation inhibition was not complete, but those various

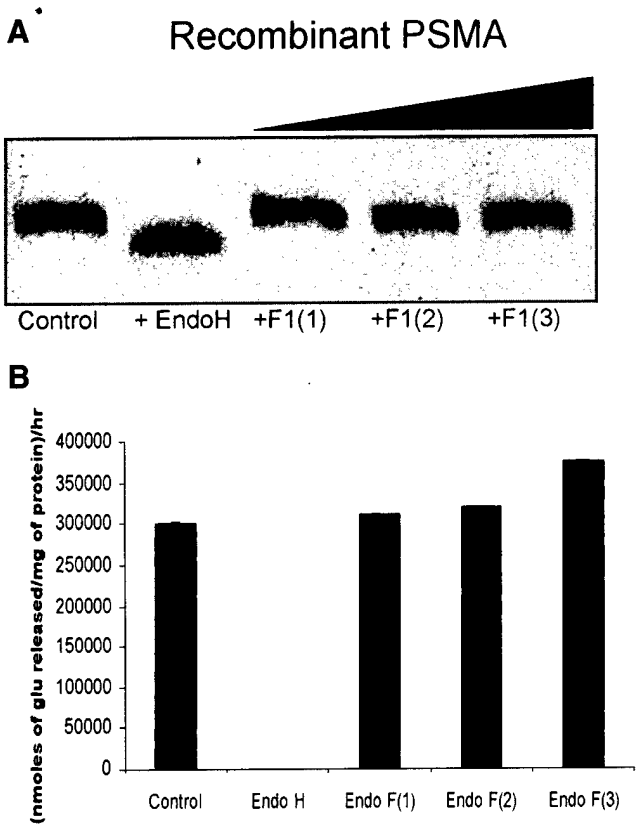

Fig. 3. Sugar attachments are important for the enzymatic activity of recombinant PSMA. The Western analysis (**A**) and folate hydrolase assay (**B**) of Endo H and different concentrations of Endo Fl (1, 2, 3 are for 16, 32, and 48 mU enzyme/0.0002 μg of protein, respectively) treated samples are shown.

PSMA-specific immuno-reactant protein species present in the whole cell extract did not have any enzyme activity (Fig. 4B, "Tun" activity profile), nor did they go to the cell surface as the membrane preparations of tunicamycin treated cells did not show any immuno-reactant PSMA band as compared to untreated PSMA control (Fig. 4A, lane 2) which again proves that deglycosylated protein does not translocate to the membrane.

Sugar Composition of PSMA Protein Varies in Different Prostate Carcinoma Cell Lines

Once we determined that sugar structure associated with PSMA protein is important for its enzymatic activity, it was important to characterize the nature of carbohydrate structures associated with PSMA protein obtained form different prostate carcinoma cells lines. To test this, the native form of PSMA protein (endogenous PSMA from membrane preparations of C4-2, CWR22Rv1, MDA PCa2b cells) was treated with a panel of endo- and exo-glycosidases: Endo H, PNGase F (cleaves between Asn and the innermost GlcNAc of glycan), β-glucosaminidase (cleaves all non-reducing terminal β-linked *N*-acetylglucosamine, β-(1–4) galactosidase (cleaves terminal Gal $\beta 1 \rightarrow 6 > 4$ 3 GlcNAc residues), Endo-*O*-glycosidase (cleaves ser/thr-linked unsubstituted Gal β (1–3) GalNAc-α-disaccharides, Sialidase (Neuraminidase, cleaves only non-reducing terminal unbranched alpha (2–3) sialic acid. This revealed a pattern, which changes with different cell types (Fig. 5). PSMA/C4-2 and PSMA/CWR22Rv1 showed sensitivity to Endo H digestion (Fig. 5, lanes A2 and C2) as well as PNGase F digestion (Fig. 5, lanes A3 and C3), whereas PSMA/MDA PCa2b showed resistance to Endo H digestion (lane B2). PSMA/C4-2 showed a band mobility shift with glucosaminidase (lane A4) indicating that it is sensitive this enzyme, but not with β (1–4) galactosidase, endo-*O*-glycosidase, sialidase (lanes A5, 6, and 7). PSMA/MDA PCa2b was sensitive to sialidase (lane B7), but resistant to other three enzymes (glucosaminidase, β (1–4) galactosidase, endo-*O*-glycosidase).

Generation of PSMA N-Glycosylation Mutants (Asn → Ala)

PSMA protein has 10 potential glycosylation sites; in order to determine which sugar attachment site is important to the enzymatic activity of the protein, we choose to mutate the putative residues in the catalytic domain of the protein (Asn^{336}, Asn^{459}, and Asn^{476}) and Asn^{638} residing in the domain F of the protein. By site-directed mutagenesis, we changed the asparagines to alanines and expressed them following transfection of PC3 cells. PC3 cells were chosen because they do not express endogenous PSMA protein. The Western blot of the whole cell extracts of the PC3 cells expressing different mutant proteins showed difference in mobility of the different mutant proteins (Fig. 6A) indicating that they have different sugar attachment or modifications associated with them. Moreover, the mutants N336A, N459A, and N476A could be detected with J591, but not the mutant N476A indicating that epitope for J591 could be the carbohydrate structure associated with the N476A. However, all four mutants could be detected with PM1T 485.5 (Hybritech), which recognizes the peptide backbone and thus detection of mutant proteins have been accomplished with PM1T 485.5 antibody. Two micrograms of protein equivalent cell extract was used for the folate hydrolase assay and demonstrates complete lack of enzyme activity of the mutants (Fig. 6B) as compared to control. Similar observation was made with NAALADase activity of the mutant proteins as well (Fig. 6C).

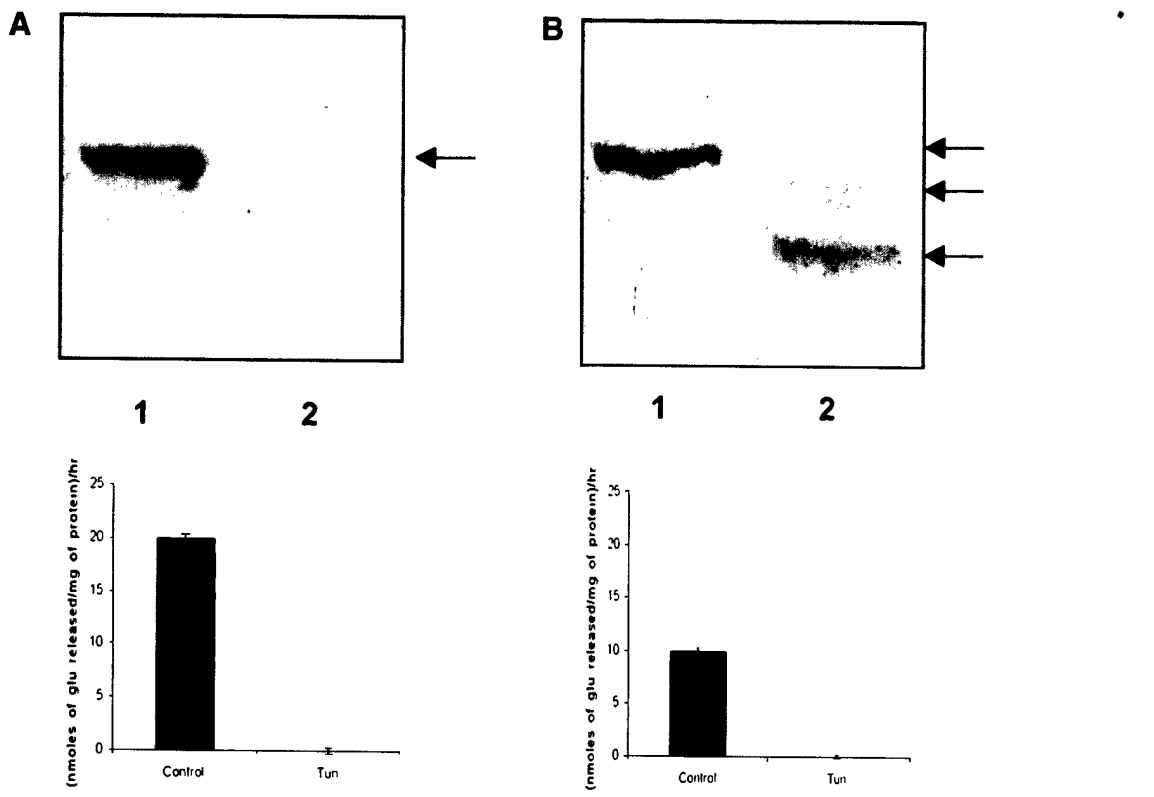

Fig. 4. Folate hydrolase assay of different membrane preparations glycosylated and deglycosylated PSMA proteins. The PC3 cells were transfected with cDNAs encoding PSMA. After 24 hr post-transfection, the cells were grown in the presence of different N-glycosylation inhibitors, Tunicamycin (10 μg/ml) for 12 hr. The cells were harvested after 36 hr post-transfection. Twenty micrograms of protein equivalent whole cell lysate or membrane preparations were analyzed by SDS–PAGE and Western blotted onto PVDF membrane. Two micrograms of protein equivalent (membrane preparations) or 20 μg protein equivalent (for whole cell extract) were taken for folate hydrolase assay. **A**: The protein analysis and the folate-hydrolase activity profile of the membrane preparations from control PC3 cells expressing WtPSMA (lane 1 for Protein expression and "control" for enzyme activity), Tunicamycin (10 μg/ml) treated PC3 cells expressing PSMA (lane 2, for protein expression and "Tun" for enzyme activity). **B**: The Western blot analysis and folate-hydrolase analysis with the whole cell extracts of the same as mentioned above.

Cellular Localization of PSMA and its Various Mutant Proteins Analyzed by Confocal Microscopy

WtPSMA exists as a cell surface glycoprotein in various cell lines. The expression of WtPSMA and its various mutants were investigated by confocal microscopy using J591 antibody (Fig. 7). We have confirmed that PC3 cells were negative with this antibody (data not shown). WtPSMA and glycosylation mutant N459A showed cell surface distribution. N476A and N638A showed localization to intracellular organelles as well. The mutant N336A was observed to be mostly intracellular with very little or barely detectable membrane expression.

Nature of Carbohydrate Structures Associated With PSMA and its Mutant Protein in PC3 Cells

In order to determine the nature of sugar residues associated with PSMA and its various glycosylation mutants, we have performed Endo H digestion of different proteins (expressed in PC3 cells) overnight and analyzed by Western blot analysis and detected by PM1T 485.5 antibody. We have found that WtPSMA, N476A, and N638A were resistant to Endo H treatment, whereas, N336A and N459A were sensitive to the Endo H digestion (Fig. 8). WtPSMA protein when expressed in PC3 cells revealed two specific closely migrating bands indicating different glycosylated forms of the protein, and upon Endo H treatment, the band pattern did not change. This indicates that the WtPSMA in PC3 cells is resistant to Endo H and composed of complex higher order sugar structures. The mutants N476A and N638A also showed two bands which remained unchanged upon Endo H treatment indicating that these two mutant proteins are also like their WtPSMA counterpart composed of complex higher order sugar structures. However, N476A undigested control band profile is different from that of WtPSMA and N638A, in having the upper glycosylated band as the predominant species, which remains unchanged upon Endo H

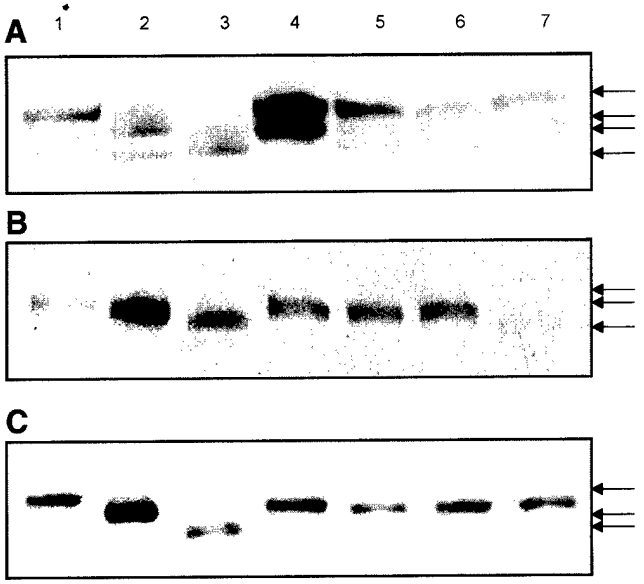

Fig. 5. Analysis of sugar composition of PSMA proteins from different prostate carcinoma cell lines. Membrane preparations from different cell types have been used which are: C4-2 (**A**), MDA PCa2b (**B**), and CWR22Rvl (**C**). Different lanes showing treatment of different membrane preparations with panel of glycosidases. They are control undigested (**lane 1**); Endoglycosidase H, 6 mU treated (**lane 2**); PNGaseF, 5 U (**lane 3**); glucosaminidase (**lane 4**); β-(1–4) galactosidase, 3 mU/sample (**lane 5**); Endo-*O*-glycosidase, 2.5 mU/sample (**lane 6**); Sialidase A, 5 mU/sample (**lane 7**). The protein samples were treated with different glycosidases overnight at 37°C, analyzed by SDS–PAGE, Western blotted onto PVDF membranes and detected with PMIT 485.5 antibody. Different glycosylated and deglycosylated PSMA bands have been shown by arrows.

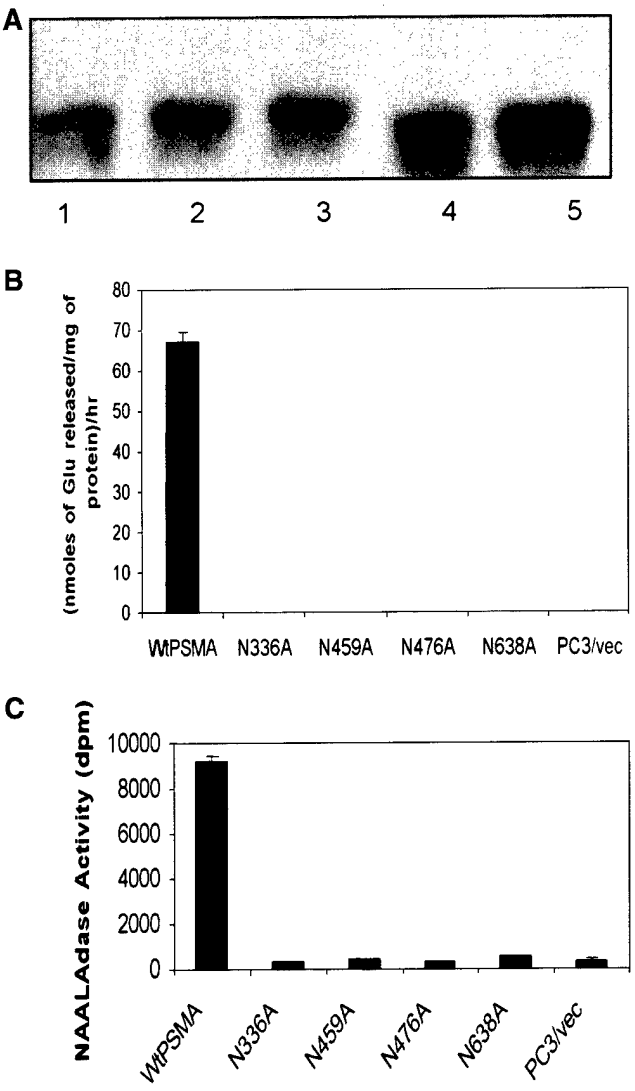

Fig. 6. Expression and analysis of different glycosylation mutant proteins. **A**: PC3 cells were transiently transfected with WtPSMA and its various mutant proteins and detected by Western analysis with PMIT 485.5 antibody. Different lanes showing different proteins, **lane 1**: WtPSMA (**2**) N336A (**3**) N459A (**4**) N476A, (**5**) N638A. **B**: Folate hydrolase profile of different mutant proteins compared to control WtPSMA and expressed in terms of (nanomoles of glu released/mg of protein)/hr. **C**: NAALADase profile of WtPSMA and different mutant proteins.

treatment. N336A showed a single species, which upon digestion with Endo H showed a lower molecular weight band indicating that it is sensitive to Endo H treatment and composed of high mannose type of sugars. N459A showed two bands, which migrated as a low molecular weight species following Endo H digestion indicating that this mutant protein is also composed of mostly high mannose type of sugars.

Biological Stability of PSMA and its *N*-glycosylation Mutants in PC3 Cells

In order to study the effect of *N*-glycosylation on protein stability, the half-life of the PSMA mutants was determined by pulse-chase experiments. Transfected PC3 cells were pulse-labeled for 1 hr with [^{35}S] methionine, then chased with an excess of unlabeled Met for various times. Following termination of the reaction, wild-type and mutant PSMA were immunoprecipated. The immunoprecipitants were resolved by SDS–PAGE and detected and quantitated by phosphorimaging. As shown in Figure 9, WtPSMA is stable and having a half-life of 55 hr. In contrast, the biological stabilities of glycosylaton mutants were dramatically reduced, but to different extents. N336A showed average half-life of 2 hr; N459A, 4 hr; N476A, 10 hr, and N638A, 55 hr.

DISCUSSION

PSMA protein which has been identified and cloned a decade ago [4,13] is a type II membrane glycoprotein, contains 10 potential *N*-linked glycosylation sites.

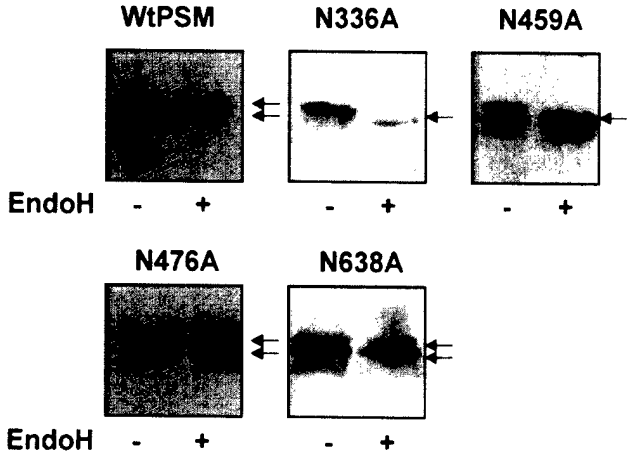

Fig. 7. Nature of carbohydrate structures of different glycosylation mutants based on their sensitivity to Endo H. Fifty micrograms of protein equivalent of whole extract of PC3 cells expressing WtPSMA or its different mutant proteins were treated with 6 mU of Endo H overnight at 37°C, and analyzed by SDS–PAGE, detected by Western blotting with PMIT485.5 antibody. The different glycosylated and unglycosylated forms of PSMA are indicated by arrows. The control untreated PSMA or its mutant proteins were indicated by "–" sign and the Endo H treated proteins were denoted by "+" sign.

PSMA has two important enzymatic functions, Folate hydrolase activity and NAALAdase activity [14]. An alternatively spliced cytosolic form of PSMA lacking *N*-terminal cytoplasmic and transmembrane regions has also been identified in normal prostatic tissue and termed as PSM′ [15,16]. It has been shown by both RNA and protein analysis that PSMA expression increases several fold cancerous tissue when compared with that in normal, tumor or benign hyperplasia [17–19]. PSMA is also present in tumor associated neo-vasculature. The reason for several fold increase in expression of membrane-bound glycosylated form over cytosolic form in advanced prostate cancer tissues is not known. PSMA is a target for therapy (based on the location of the protein in prostate cancer and tumor associated neovasculature of other solid tumors). In this context, it is very important to study the importance of sugar structures associated with PSMA protein, its relationship to the folate hydrolase function of protein and its role in progression of prostate cancer. In our present study we have focused on the folate hydrolase function of PSMA and the role of sugar moieties on the novel enzymatic functions of this protein. We have used the native form of PSMA protein as it is expressed endogenously in various prostate carcinoma cell lines, ectopically expressed PSMA in PC3 cells (PC3 cells normally do not express PSMA) as well as recombinant PSMA protein (44-750 amino acids) expressed in CHO cells as our protein source. Endoglycosidase H sensitivity/resistance of PSMA protein revealed that PSMA is mostly composed of N-linked sugars, which are of

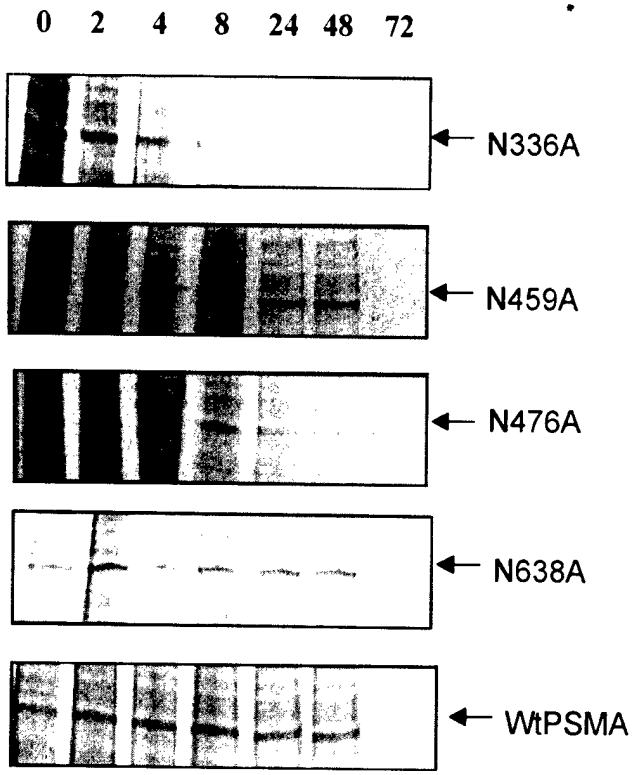

Table1:

Protein	Half life
WtPSMA	55 hrs
N336A	2 hrs
N459A	4 hrs
N476A	10 hrs
N638A	55 hrs

Fig. 8. Biological stability of PSMA and its different mutants. PC3 cells transiently transfected with different cDNAs encoding WtPSMA or its different mutants were pulsed with ^{35}S methionine containing medium for 1 hr followed by chase with medium supplemented with cold methionine and harvested after different periods of time, namely 0, 2, 4, 8, 24, 48 hr, respectively. The samples were immuno-precipitated with PMIT485.5 antibody and ran by SDS–PAGE and analyzed by phosphoimager analysis. Different band intensities were measured to determine the half-lives of different proteins (Table I).

high mannose type or fucosylated hybrid mannose type. Apparent Endo F1 resistance of PSMA protein indicates that the sugar structures are mostly the fucosylated hybrid mannose type of linkage. Endo H can hydrolyze fucosylated hybrid mannose type of sugar with equal efficiency as high mannose type. However core-linked fucose reduces the hydrolysis rate of Endo F1 by over 50-fold relative to high mannose structures [12]. This shows that PSMA in C4-2 cells is composed of

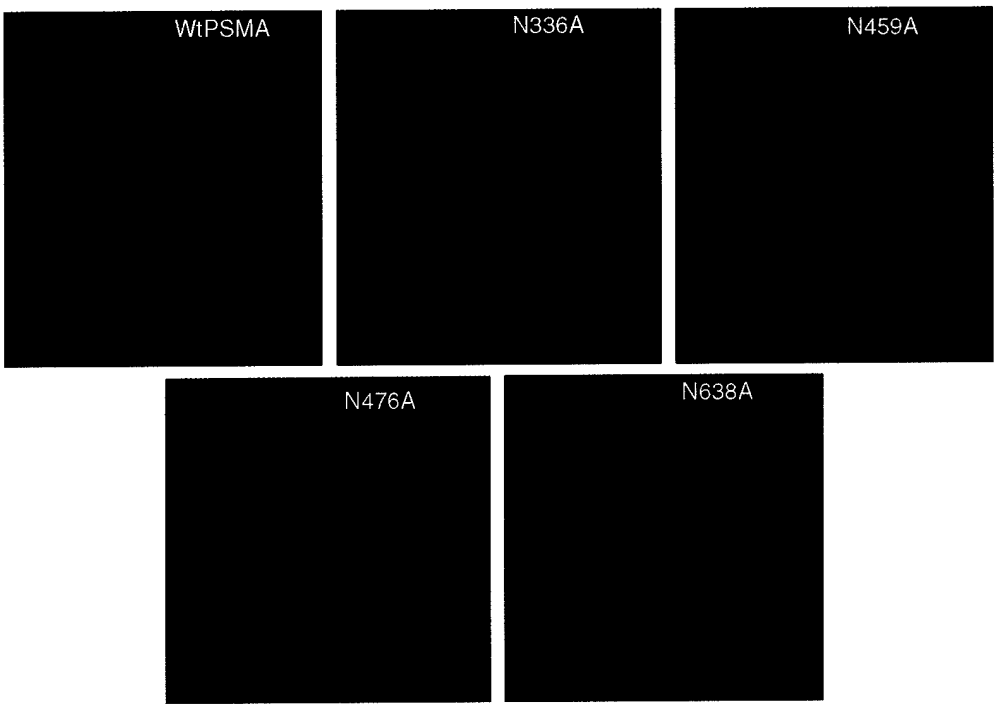

Fig. 9. Immunofluorescence microscopy of different mutants expressed in PC3 cells. The PC3 cells (grown on coverslips) transiently transfected with PSMA and its various mutants were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by permeabilizing with PBS containing 0.2% Triton X-100 for 10 min at room temperature. The coverslips were washed with PBS three times and blocked with 3% BSA, 5% goat serum in TBST (0.05 M Tris-HCl, pH 7.6; 0.138 M NaCl, KCl 0.0027 M), followed by incubation with primary antibody PMIT 485.5 in 3% BSA in TBST (Tris buffered saline with 0.05% Tween-20). The coverslips were visualized with secondary antibodies, biotinylated-goat-anti-mouse antibody, and streptavidin-FITC subsequently. The coverslips were mounted on vectashield and visualized under confocal microscope.

fucosylated hybrid mannose type of glycans. Removal of sugar residues abolishes PSMA's enzymatic activity completely, whereas the control untreated protein incubated with Endo H or Endo F1 buffer did not inhibit enzyme activity (Fig. 2). The same observation was true for the recombinant secretory form of PSMA protein, which showed that in CHO cells, PSMA protein is composed of fucosylated hybrid mannose type of sugars (Fig. 3). The reason for the apparent 25% increase in the folate hydrolase activity of the recombinant protein treated with 48 mU of Endo F1 as compared to control buffer treated protein is not known. Endo F1 (at high concentration) has probably cleaved the fucosylated recombinant PSMA Protein partially (a slightly faster mobility of the protein band compared to control) resulting in opening the substrate pocket. The sensitivity of PSMA protein from other prostate carcinoma cell lines to the treatment with different glycosidases was found to be different. For example, PSMA/C4-2, PSMA/CWR22Rv1 was found to be sensitive to Endo H, indicating that it could be composed of either high mannose type or fucosylated hybrid mannose type sugars (Fig. 5). Unlike PSMA/C4-2, PSMA/MDA PCa2b (Fig. 5), PSMA expressed in PC3 (Fig. 8) cells were composed of complex higher order structures (based on their resistance to Endo H). PSMA/MDA PCa2b cells are sensitive to sialidase indicating that terminal sialic acid structures are present. Lack of cleavage by endo-*O*-glycosidase indicates that *O*-linked structures are not present. Resistance to β (1–4) galactosidase indicates that polylactosamine structures are not present. PSMA protein from all cell types that we have tested so far, are sensitive to PNGaseF leading to complete deglycosylation of the protein leaving a 85 kDa protein band which is equivalent to the predicted deglycosylated protein form of PSMA indicating the bulk of the sugar attachment is N-linked. In addition, these cells also have other sugar modifications as well. We have found that J591 antibody [6] could not detect the completely deglycosylated form of the protein (complete removal of sugar by PNGase F digestion), indicating that epitope for J591 could be the sugar structure associated with the protein. The deglycosylated protein could be detected with PM1T 485.5 antibody. For all our protein analysis we have used the PM1T 485.5 antibody, which recognizes the peptide backbone of the protein. It will be important to analyze whether prostate cancer cells are glycosylating PSMA differently than that of normal prostate or endothelial cells expressing PSMA in other solid tumors.

N-linked sugars are important for the folate hydrolase activity of the PSMA is further established by synthesizing the protein in presence of N-linked glycosylation inhibitor, tunicamycin, which showed that protein synthesized are defective in glycosylation [95 kDa (minor) and 85 kDa (major)] are completely inactive in enzymatic function. Moreover, such defective glycosylated forms of PSMA do not make it to the cell surface.

Once we established that PSMA is composed of N-linked sugars and that the sugar residues are important for its enzymatic activity, and that different prostate carcinoma cell lines composed of different type of sugar structures, it was pertinent to study the structure-function of PSMA protein in terms of its glycosylation. We also wanted to determine which sugar attachment sites are important for the enzymatic function of protein. With this goal in mind we have mutated the Asn residues to ala at the catalytic site of the protein (Asn^{336}, Asn^{459}, and Asn^{476}) and found that the mutation of any of them abolishes the enzymatic activity of the protein completely (both folate hydrolase and NAALADase activities). N459D mutant has been reported to change in migration pattern and specific activity of the protein compared to Wt control [20]. We have mutated another potential glycosylation site N638A and found that this mutation also abolishes the folate hydrolase and NAALADAse activities of the protein. Asn^{638} is located in a domain adjacent to the catalytic domain of the protein (domain F), which probably changed the conformation of the protein making it enzymatically inactive.

Further biochemical analysis of mutant proteins revealed more dramatic changes on the physiological properties and the function of the proteins. The mutations in sugar attachment sites dramatically alter their biological stability (Fig. 9). Previously, biological stability of PSMA had not been determined. We have expressed WtPSMA or the mutant proteins into PC3 cells by transient transfection and found that the absence of only one of the N-glycosylation in the catalytic site was sufficient to reduce the stability of the protein. Wt protein has a half-life of 55 hr whereas the mutant proteins have dramatic reduction in their half-lives (2 hr for N336A; 4 hr for N459A; and 10 hr for N476A) except for the domain F glycosylation mutant N638A. Although, N638A mutant showed lack of enzymatic activity, the biological stability of this protein was comparable to that of Wt protein (55 hr). Apart from lack of enzymatic activity, N638A protein showed all characteristics of Wt protein, in terms of stability, Endo H resistance, cellular localization apart from perturbing the specific conformation of active-site, other structural parameters of this protein remained like WtPSMA. Mutant proteins also showed variation in terms of their Endo H sensitivity, N336A and N459A are retained in the ER, whereas N476A and N638A are resistant to Endo H, indicating that they have complex carbohydrate structures.

Impact of N-glycosylation site mutations on the stability of proteins have been reported in several instances, for example, mannose-6-phosphate receptor [21], transferrin receptor [22] and acetylcholinesterase [23], and Marpin A [24]. It could be possible that oligosaccharide side chains protect the protein against protease-mediated degradation [(e.g., Interferon-γ, glycans at Asn^{25} is important for protease resistance [25]]. Another possible explanation could be that the mutations affect the folding and oligomerization process, resulting in prolonged stay in ER and an increased proteolytic breakdown in this compartment which could explain the dramatic reduction in the stability of PSMA mutant proteins.

Analysis of the carbohydrate structures of PSMA protein from LNCaP cells and in vivo specimens have been reported by Holmes et al. [16] where it has been shown by endo- and exo-glycosidase analysis that PSMA is mainly composed of high mannose kind of sugars. We have extended these observations and analyzed in terms of enzymatic activity of protein and found that sugars are important for the enzymatic activity of the protein. Our analysis also shows that unglycosylated or partially glycosylated versions of PSMA proteins are not enzymatically active (lower molecular weight forms produced in tunicamycin treated cells), which also makes a possibility that alternatively spliced isoform of PSMA protein, PSM' is enzymatically inactive. Barinka et al. [26] has expressed truncated cytosolic version PSMA protein and found it to be enzymatically inactive. Likewise detailed analysis of carbohydrate structures associated with PSMA protein in different cancer tissues, tumor neovasculature will help us understand the biology of prostate cancer better as it progresses into metastasis and will pave the way for developing better targeting agents.

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Tumor Target Prostate Specific Membrane Antigen (PSMA) and its Regulation in Prostate Cancer

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Abstract Prostate specific membrane antigen (PSMA), is a unique membrane bound glycoprotein, which is overexpressed manifold on prostate cancer as well as neovasculature of most of the solid tumors, but not in the vasculature of the normal tissues. This unique expression of PSMA makes it an important marker as well as a large extracellular target of imaging agents. PSMA can serve as target for delivery of therapeutic agents such as cytotoxins or radionuclides. PSMA has two unique enzymatic functions, folate hydrolase and NAALADase and found to be recycled like other membrane bound receptors through clathrin coated pits. The internalization property of PSMA leads one to consider the potential existence of a natural ligand for PSMA. In this review we have discussed the regulation of PSMA expression within the cells, and significance of its expression in prostate cancer and metastasis. J. Cell. Biochem. 91: 528–539, 2004. © 2003 Wiley-Liss, Inc.

Key words: prostate specific membrane antigen; carboxypeptidase; folate hydrolase; folylpoly-γ-glutamate; dileucine motif; internalization

Prostate cancer represents an excellent target, especially for monoclonal antibody therapy for a number of reasons that include [Ma et al., 2003].

1. The prostate is a non-essential organ and its destruction will not harm the host and the identification of tissue specific antigens for antibody development is easier than elusive tumor specific antigens.
2. The sites of prostate metastasis being lymph nodes and bone are sites that receive high levels of circulating antibodies.
3. Metastases are typically of small volume allowing ready access to therapy and are identified early following primary therapy by elevation in serum PSA.
4. The PSA serum marker provides a means to monitor therapeutic response.
5. Patients at high risk for subsequent failure from primary therapy can be readily predicted enabling initiation of therapy while the tumor burden is minimal.

PROSTATE SPECIFIC MEMBRANE ANTIGEN

The tissue specific protein, prostate specific membrane antigen (PSMA) is an excellent target for imaging and therapy because, it is a cell surface protein which presents a large extracellular target, and it is expressed at levels that are about a thousand-fold greater than the minimal expression seen in other tissues such as kidney, proximal small intestine, salivary gland. Some minimal expression is also observed in the brain, but most agents and especially antibodies do not penetrate the brain because of the blood–brain barrier. While the initial antibody used for imaging PSMA, capromab pentetide, is the only prostate cancer imaging agent approved by the FDA, it has had problems in terms of specificity and sensitivity. The poor quality of the initial antibody was felt to be due to the fact that it recognizes an internal epitope of the protein and is binding to areas of tumor necrosis, and necrosis is less likely in areas at metastatic sites in the

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bone. Second generation antibodies such as the humanized version of J591 developed by Dr. Bander at Cornell Weil School of Medicine in New York has been in clinical trials and has demonstrated an ability to image all sites of metastasis, especially bone with nearly 100% specificity and sensitivity. Other non-prostate sites of minimal expression are not imaged. Other companies are also developing second-generation antibodies such as the cytogen/progenics joint venture company developing fully human antibodies to the external domain of PSMA. The results of these different groups using these second generation antibodies are very encouraging and are demonstrating therapeutic activity in delivering radionuclides and cytotoxic agents resulting in therapeutic responses in both preclinical and early clinical trials [Bander et al., 2003]. An unanticipated site of PSMA protein expression is that it is found to be strongly expressed in the neovasculature of all solid tumors but not normal vasculature and as such will serve as an imaging and therapeutic target for all solid tumors.

PSMA has an important role in prostate carcinogenesis and progression, glutamatergic neurotransmission, and folate absorption [O'Keefe et al., 2001]. Each of these different areas of research activity leads to different names being given to PSMA. Because of its strong expression in the prostate (where its function is unknown), it is named as PSMA; in central nervous system, where it metabolizes the brain neurotransmitter, *N*-acetyl-aspartyl-glutamate, it is named NAALADase; in the proximal small intestine its role is removing gamma-linked glutamates from poly-γ-glutamated folate, folate hydrolase FOLH1, and as a carboxypeptidase, glutamate carboxypeptidase II, GCPII. Our focus in this review will be on its biology and role in the prostate and prostate cancer. PSMA is up-regulated many fold in prostate cancers (PCA), metastatic disease, and hormone-refractory PCAs. PSMA expression is modulated inversely by androgen levels [Israeli et al., 1993; Wright et al., 1996]. Most interestingly, PSMA expression has been found in the neovasculature of most of the solid tumors (not in vasculature of the normal tissues) [Silver et al., 1997]. The exact significance of this is not known. However, due to it's intriguing yet unexplained distribution, PSMA can serve as a detecting agent for metastatic foci of primary cancer. PSMA can be used as a target of imaging agent to detect metastatic tumor site, or PSMA can also be used to detect prostate cells in the circulation or lymphatics. PSMA serves as a target for the delivery of therapeutic agents such as cytotoxins or radionuclides.

PSMA Discovery and Mapping

PSMA is a type II membrane glycoprotein, Mr~ 100,000 dalton with an intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750) (Fig. 1A). Human *PSMA* gene was first cloned in Dr. Heston's laboratory from LNCaP cells [Israeli et al., 1993] and was found to be located in chromosome 11p11-12, which encodes for PSMA transcript expression in prostate [Leek et al., 1995; Rinker-Schaeffer et al., 1995; O'Keefe et al., 1998]. Another gene, highly homologous to PSMA was found to be located at the loci 11q14.3 is called PSM-like. The PSM-like gene is expressed in different tissues, such as kidney and liver, but not in prostate [O'Keefe et al., 2001].

Variants of PSMA

PSMA is alternatively spliced to produce at least three variants (Fig. 1B), most important of which is PSM', the cDNA of which is identical to PSMA except for a 266-nucleotide region near the 5′ end of PSMA cDNA (nucleotides 114–380) which codes for the transmembrane region of the protein. PSM' is therefore located in the cytoplasm. Su et al. [1995] used RNAse protection assays to examine the expression of PSMA and PSM' in normal versus benign prostate hyoperplasia (BPH) versus prostate cancers. They found increasing expression of PSMA in tumors relative to normals and generated a tumor index based on PSMA: PSM' ratio which is 9–11 in LNCaP cells, 3–6 in prostatic carcinoma, 0.75–1.6 in BPH, and 0.075–0.45 in normal prostate. The other two variants of PSMA are PSM-C (with transcription start site same as PSMA, splice donor site same as PSM', different splice acceptor site located within intron 1, includes a novel exon 1b), which is identical to PSM' and PSM-D (same donor site as PSM', but acceptor site includes novel exon 1c), has a new translation start site followed by 42 novel amino acids with a motif of AAYACTG-CLA (found in growth factor cys-knot family of

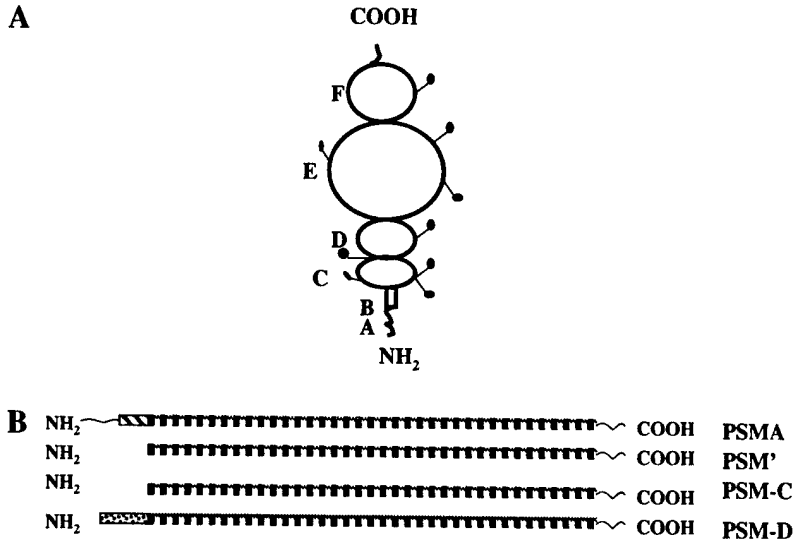

Fig. 1. **A**: Showing the globular nature of prostate specific membrane antigen (PSMA) protein with different domain. Domains A: Amino acids 1–19, intracellular, cytoplasmic; B: 20–39, transmembrane; C: 40–144; D: 173–248; E: 275–596; F: 597–756. There are two linker regions between domains C and D (amino acids 145–172) and domains D and E (amino acids 249–274). Domain C, D, E, and F are the extracellular domains. Domain E is the catalytic domain with the zinc binding and substrate binding sites. **B**: Schematic view of the different splice variant of PSMA detected in LNCaP cells. PSM' lacks the intracellular cytoplasmic domain and transmembrane domain, the rest is identical to PSMA. PSM-C, although produced by alternative splicing, has a different splice acceptor site than PSM', encodes a protein identical to PSM'. PSM-D, has a unique 42 amino acid long domain at N-terminus and rest is identical to PSM'.

proteins) and rest of the PSMA protein in frame [O'Keefe et al., 2001]. The implication of such alternative splice variants in prostate cancer cells is not known at present.

Unique Enzymatic Functions of PSMA

PSMA is a protein with two unique enzymatic functions, including NAALADase activity (cleaving terminal glutamate from the neuropeptide, *N*-acetyl-aspartyl-glutamate, NAAG) and folate hydrolase activity, which cleaves the terminal glutamates from γ-linked poly glutamates. NAAG is concentrated in neuronal synapses while folylpoly-γ-glutamates are present in dietary components and PSMA protein of the surface of the brush border surface of small intestine enables the generation of folates and subsequent folate uptake. So the question that comes in one's mind is what is a protein like PSMA with such interesting activity profile doing on the surface of prostate cells and why is its expression level enhanced so many fold in prostate cancer cells? The answer is still unknown but there are several possible explanations, which we will discuss in this review.

The structural similarities between PSMA and other proteins are known. The *PSMA* gene is highly homologous to neuropeptidase, NAALADase, which releases neurotransmitter glutamate from neuropeptide NAAG; human glutamate hydrolase, which is capable of folylpoly-γ-glutamate hydrolysis [Yao et al., 1996]; I100 (human dipeptidyl peptidase IV), associated with apical brush border of intestinal epithelial cells [Darmoul et al., 1992; Shneider et al., 1997]. Human prostate PSMA (FOLH1) and rat NAALADase is classified as GCPII, a member of M28 peptidase family of metalloproteases [Rawlings and Barrett, 1997], with the residues conserved for Zn^{2+} and substrate binding. Human PSMA has about 91% homology to mouse PSMA (folh1) [Bacich et al., 2001]. Human, mouse, rat, and porcine folylpoly-γ-glutamate carboxypeptidases have 10, 10, 9, and 12 putative glycosylation sites, respectively [Israeli et al., 1993; Bzdega et al., 1997; Halsted et al., 1998; Ghosh, 2003]. PSM' and PSM-like have nine and five potential glycosylation sites, respectively. Mouse, rat, and pig homologs have an additional glycosylation site, which is not present in human forms. Glycosylation of PSMA plays important role in its targeting the protein to the cell membrane, proper protein folding, and enzymatic activity. Removal of sugar residues partially or completely (enzymatically or by mutagenesis) abolishes the enzyme activity of the protein [Ghosh, 2003]. More interestingly, the glycosylation profile of PSMA

obtained from different prostate cancer cell lines was found to be different, leading one to speculate that the different sugar epitopes may play a role in metastasis of prostate cancer. Further work has been carried out in the laboratory to address such question. Human PSMA has homology to human transferrin receptor (Tfr). Both are type II glycoproteins and PSMA shares about 54% homology to the Tfr and about 60% with transferrin receptor 2 (Tfr2) [Kawabata et al., 1999]. The Tfr exists in dimer form due to interstrand sulfhydryl links. PSMA exists in dimeric/monomeric form. Recombinant protein of the extracellular domain of PSMA also exists in readily interconvertible dimer–monomer forms. PSMA is expressed as non-covalent homodimer on the surface of LNCaP cells as well as on the 3T3 cells stably transfected with full-length PSMA [Schulke et al., 2001]. Like the Tfr, PSMA can undergo internalization. It is yet to be characterized whether the dimer or monomer internalize. It is not understood what induces dimer formation or cause dissociation to the monomer form. The difference is important because the dimer has enzymatic activity and the monomer does not have enzymatic activity.

Endocytic Function of PSMA

PSMA, like other cell surface receptors undergoes internalization constitutively and such spontaneous internalization is enhanced three-fold in a dose dependent manner by PSMA specific monoclonal antibody J591 [Liu et al., 1998]. It has been shown very clearly biochemically (by using biotinylated cell surface PSMA followed by internalization of the protein), by immuno-fluorescence analysis or immuno-electron microscopy, that PSMA or PSMA–antibody complex undergoes internalization through clathrin coated pits and closely resembles the internalization pathway of Tfr and finally ends up in the lysosomes. Such constitutive internalization of PSMA may reflect the recycling of a structural protein or may be mediated by binding of a ligand. A detailed characterization of antibody mediated PSMA internalization revealed the resemblance with EGFR receptor with its ligand [Haigler, 1983]. It is well known that many ligands and their transmembrane receptors are internalized through clathrin coated pits (receptor mediated endocytosis). Formation of antibody–antigen complexes on the cell surface often results in internalization through a pathway closely resembling the receptor mediated endocytosis of peptide hormones, growth factors, and natural ligands [Pastan and Willingham, 1981] (Fig. 2). It can be speculated from these findings that PSMA may have a transport function for an yet unidentified ligand. Monoclonal antibody such as J591 acts as surrogate ligand inducing internalization.

Targeting internalization of receptors through coated pits and their traffic through endocytic compartments are mediated through specific signals or motifs located on the cytoplasmic tail of the receptors. There are two major class of sorting signals which mediate internalization of membrane proteins from plasma membrane and sort these proteins to endosomes/lysosomes and finally to the compartment for peptide loading [Sandoval and Bakke, 1994; Marks et al., 1997; Nordeng et al., 1998]. These sorting signals are tyrosine based motifs NPXY and YXXΦ motifs [Marks et al., 1997] and dileucine motif [Sandoval and Bakke, 1994]. Tyrosine motifs are identified in a variety of receptor molecules like the Tfr, low density lipoprotein receptor, and asialoglycoprotein receptor [Trowbridge et al., 1993]. Dileucine (or leucine–isoleucine sequence) motif (Fig 3A) is important for internalization and lysosomal targeting were found in the γ-δ chain of T cell receptor, CD4, IFN γ [Shin et al., 1991; Letourneur and Klausner, 1992; Farrar and Schreiber, 1993]. Tyrosine based motifs interact with adaptor complexes AP1, AP2, and AP3 [Traub and Kornfeld, 1997; Hirst and Robinson, 1998] and dileucine based motifs bind to the β subunits of AP1 and AP2, μ chains of AP1 and AP2 have also been reported to bind to these signals. Apart from this, leucine based signals of lysosomal protein LIMPII and melanosomal membrane protein tyrosinase have been shown to bind to AP3 [Honing et al., 1998]. PSMA has a dileucine motif present at its cytoplasmic tail. Mutation of first leucine (Leu 4) did not change the internalization of mAb J591, in contrast, conversion of second leucine (Leu 5) resulted in complete lass of internalization indicating this leucine is important for the internalization [Rajasekaran et al., 2003; Ghosh and Heston, 2003b]. This implied that the dileucine motif is responsible for the internalization. However, the dileucine motif is associated with the basolateral targeting of protein and PSMA is found at the apical surface of the cell. In case of

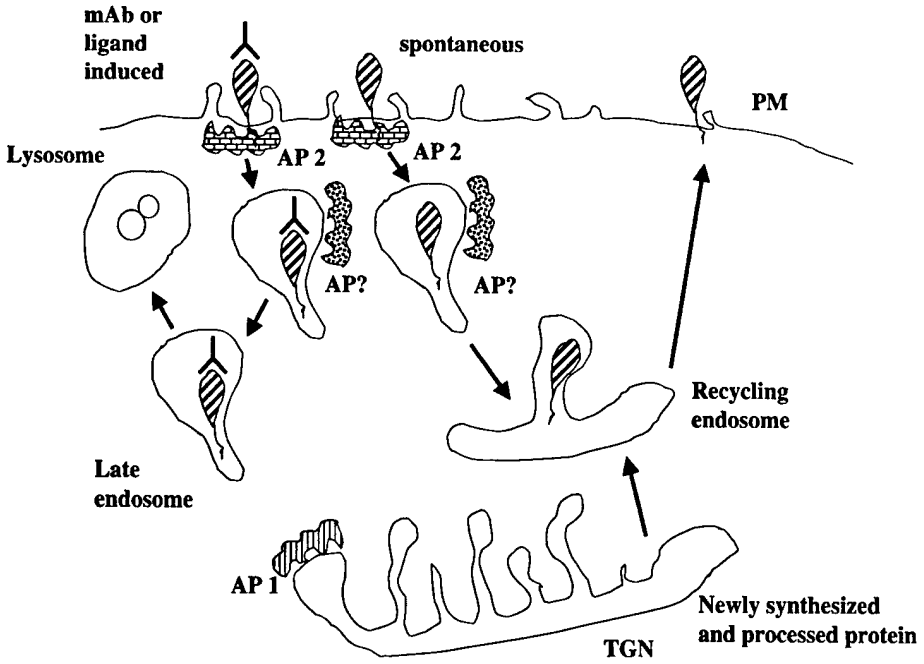

Fig. 2. Showing the spontaneous or antibody induced internalization of PSMA through clathrin coated pits. The proteins can either recycle through recycling endosomal compartment (REC) and go to the plasma membrane or they can go to the lysosomes through late endosomes. The cytoplasmic tail of PSMA contains an internalizaton signal, which enables it to internalize into endosomal vesicles.

PSMA, the first amino acid methionine located five amino acids up-stream of the crucial leucine is involved in the internalization, which makes this signal a unique internalization signal "MXXXL" in PSMA. Amino acid residues adjacent to such motifs has been shown to influence its function. Dileucine signal of CD4 is active when adjacent serine residues are phosphorylated [Pitcher et al., 1999]. Cytoplasmic tail of PSMA has consensus protein kinase C sequence (Thr-14) and has two other hydroxyl containing residues (Thr-8, Ser-10), that might serve as phosphorylation acceptor sites. It remains to be seen how mutation of such residue affect the internalization function of the protein. A detailed mutational analysis has been carried by Rajasekaran's group, which did not have any effect on internalization function of PSMA. It is known at least in EGFR [Kil et al., 1999], that the dileucine motif and its neighboring residues need to form an amphipathic helix with hydrophilic residues pointing towards one surface, and hydrophobic residues pointing towards the other for interaction with adaptor proteins needed for sorting. The predicted protein structure of PSMA N-terminal cytoplasmic tail showed that this region (residues N^3 to R^{19}) has a probability to take up a α helical structure and helical wheel projection of this region showed that this helix is an amphipathic α helix with hydrophobic residues projecting towards one surface and hydrophilic residues on the other (Fig. 3B).

A cytoplasmic leucine based motif has been shown to be involved in the lysosomal targeting of several membrane proteins [Letourneur and Klausner, 1992; Haft et al., 1994; Dittrich et al., 1996; Kil et al., 1999]. PSMA colocalizes with lysosomal marker Lamp1 in LNCaP cells (endogenously express PSMA) or Cos cells expressing transfected PSMA [Rajasekaran et al., 2003] or PC3 cells ectopically expressing PSMA [Ghosh and Heston, 2003b] indicating PSMA is being localized within lysosome. Furthermore swapping the MWNLL (the first five amino acid of PSMA sequence) and MWNLA (fifth leucine has been changed to alanine) to Tac antigen, this group has shown that wild type motif could transport the Tac antigen (which is not a lysosome resident) to the lysosome, whereas mutant motif could not, indicating that MXXXL signal in PSMA is indeed a lysosomal signal. Knowledge of PSMA's internalization function and regulation can be exploited in cancer therapeutics. A detailed analysis is required to define the putative natural ligand for internalization,

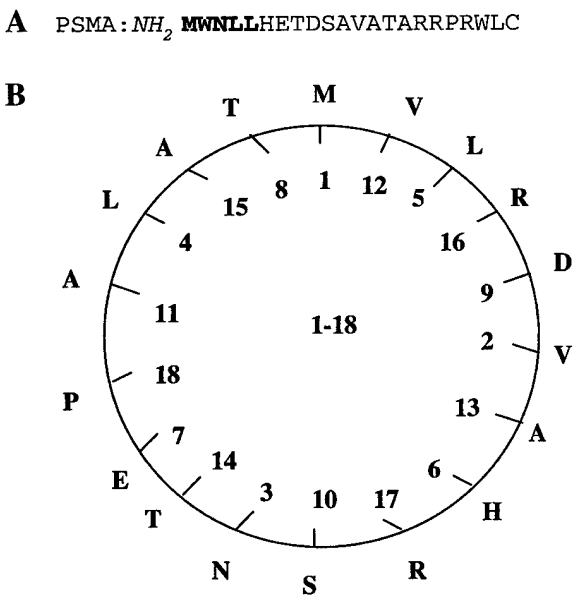

Fig. 3. PSMA has a dileucine motif at its C-terminal tail. Dileucine motifs serve as lysosomal targeting signal and usually reside within the cytoplasmic tail of the protein (either N- or C-terminal). **A**: Showing the peptide sequence N-terminal cytoplasmic sequence here with putative internalization motif shown in bold. The actual internalization signal of PSMA is the first five amino acids MXXXL. **B**: The helical wheel projection of the C-terminal tail region (N-terminal 19 amino acid). By using the predict protein program (http://cubic.bioc.columbia.edu/predict-protein), it was found that the N-terminal region contains a α-helical region. Using this sequence, we could make a helical wheel projection by using the site http://www.site.uottawa.ca/~turcotte/resources/helixwheel and found that the one face of the helix contains the hydrophobic residues and the other face of the helix contains the hydrophilic residue, indicating that this region is important for protein–protein interaction.

and what role it plays on the biological function of PSMA. Our lab has shown that substrates and antagonists of the carboxypeptidase function do not alter the rate of internalization, which shows that internalization and enzymatic function are two independent processes. Furthermore, it will be interesting to find out if this natural ligand for internalization could substitute for the mAb in a targeted therapy approach.

REGULATION OF PSMA EXPRESSION AND ITS IMPLICATION IN PROSTATE CANCER

PSMA–Filamin Interaction

Recently it has been shown that cytoplasmic tail of PSMA interacts with actin binding protein Filamin a (FLNa) [Anilkumar et al., 2003]. PSMA's association with FLNa is necessary for its localization to recycling endosomal compartment (REC). In filamin negative cells, the internalized PSMA accumulates in diffused vesicles throughout the cytoplasm. This distribution can be altered by introducing FLNa in this cell and the proteins localize into REC. PSMA–FLNa interaction decreased its rate of internalization by 50%. It could be that linking of PSMA with actin cytoskeleton by FLNa keeps the proteins attached to cell membrane, and eliminates their ability to bind adaptor proteins, hence reduction in internalization rate. A dissociation from FLNa might help in binding of PSMA cytoplasmic tail with adaptor proteins, which leads to endocytosis. Therefore, FLNa and adaptor proteins could be competing for binding at the same site of PSMA. Internalization motif mutants that could not undergo endocytosis, but could bind very srongly to PSMA support this theory. Figure 4 gives a summary of this event. The importance of phosphorylation of PSMA protein at certain putative phosphorylation sites and its implication on its binding with FLNa remains to be solved.

Regulation of PSMA Expression by PSMA Enhancer (PSME)

PSMA has been shown to be severalfold increased expression in prostate cancer; its expression is suppressed by androgen. Currently, two regulatory elements controlling PSMA expression have been characterized. The proximal 1.2 kb PSMA promoter and PSME [O'Keefe et al., 1998], located within the third intron of FOLH1, rendering prostate specific expression of PSMA. PSME is activated in prostate specific manner, negatively regulated by androgen receptor (AR) and its expression is up-regulated in prostate cancer. Detailed study showed that proximal 90 bp of PSME contained enhancer element with an AP3 site responsible for elevation of promoter activity of PSME beyond the basal level [Lee et al., 2002]. Furthermore, recent work by this group has shown that Ca^{2+}-dependent activation PSME transcription factor NFATc1 isoform binds to AP1. In presence of Ca^{2+}, NFATc1, protein gets dephosphorylated through Ca^{2+}-dependent calcineurin, which drives the translocation of NFAT protein to nucleus and activates transcription of PSMA.

Direct repeat regions of PSME harbors nine copies of SRY/SOX sites. SRY, SOX 7, and SOX

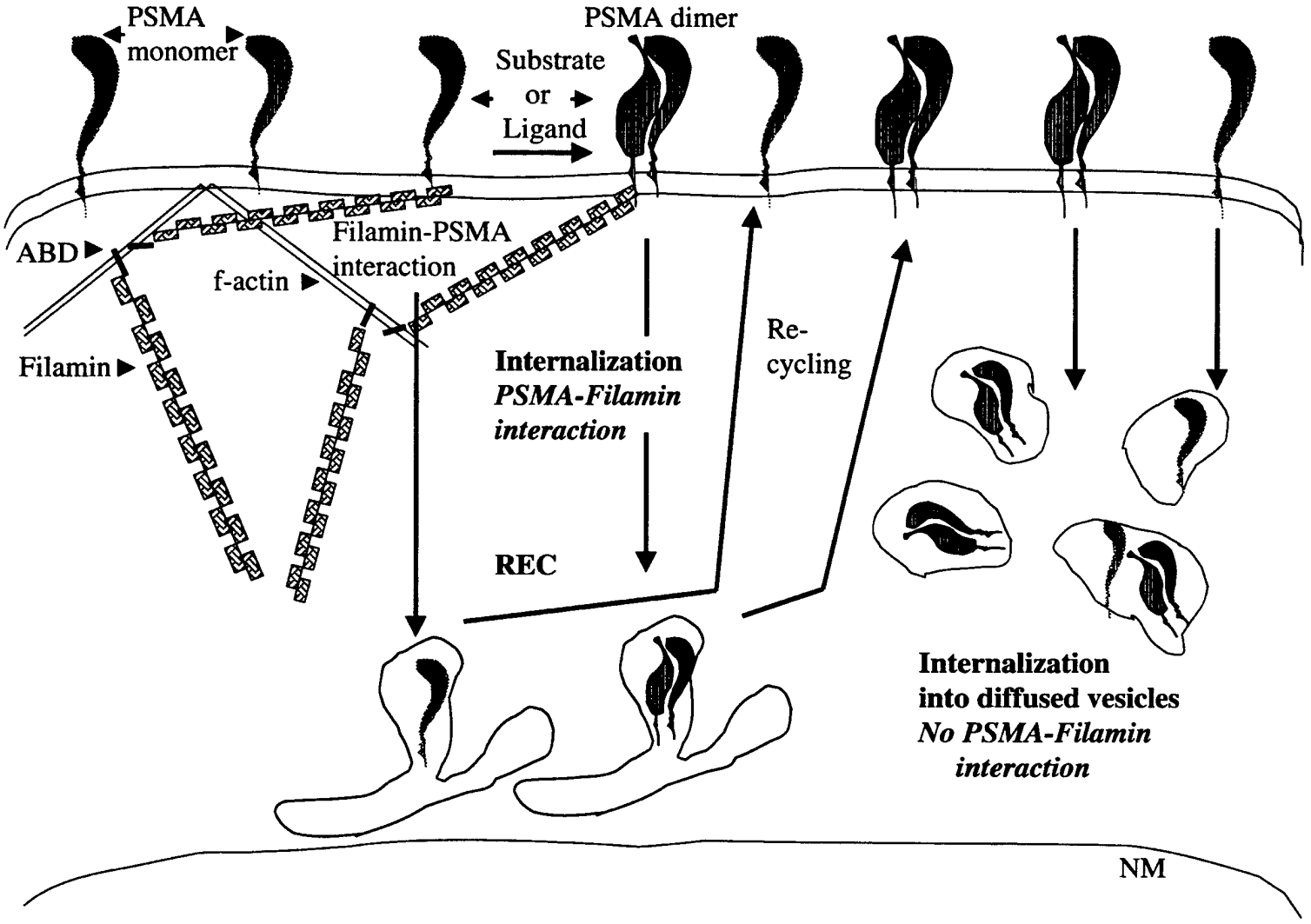

Fig. 4. A schematic diagram showing the PSMA–Filamin a (FLNa) interaction within the cells and how such interaction modulates PSMA function. Which form of PSMA undergoes internalization (monomer or dimer form) is not yet known clearly. In presence of FLNa, PSMA undergoes internalization to the REC located at the perinuclear region. In absence of FLNa, PSMA is distributed through the diffused vesicle throughout the cytoplasm.

18 are reportedly expressed prostate cancer and prostate epithelial cells [Takash et al., 2001]. SRY or SOX may interact with androgen receptor DNA binding domain (ARDBD), and as a result AR sequesters these tissue specific proteins, causing repression of PSME, which could partially explain AR mediated repression of PSME.

Ca^{2+} ions positively regulate PSMA expression. Ca^{2+} influx probably takes place through CaT-like calcium channel, which is strikingly correlated with the malignancy of prostate cancer as well as PSMA expression [Wissenbach et al., 2001]. But how could that start? The possible explanation may involve glutamate receptors. We have observed metabotropic glutamate receptors by gene array analysis of LNCaP cells (Heston et al., unpublished observation). In prostate cancer cells, such receptors could get activated constitutively by free glutamates (an agonist to such receptor) released as a by-product of folate hydrolase/NAALADase action of PSMA expressed on the cell surface (PSMA level is up-regulated many fold in prostate cancers) and can modulate the function of potassium and calcium channels, which might cause change in resting membrane potential. Such change in membrane potential could cause oxidative damage to the cells, causes release of Cl^- ions [Shuba et al., 2000] and continuous influx of Ca^{2+} ions through calcium channels (to compensate for such change in membrane potential).

Interaction of AR–FLN a

AR, is a member of steroid/ nuclear receptor superfamily, mediates male morphogenesis in utero, gametogenesis, and prostate growth in older man. It has four principle domains, large N-terminal transactivation domain (ARTAD), ARDBD, a hinge region, and a C-terminal ligand binding domain (ARLBD). In absence of androgen, AR is cytoplasmic. Androgen binds specifically to a ligand binding pocket in the lower half of LBD, causing conformational change. Ligand-AR goes to nucleus, where ARDBD interacts with specific response elements on the promoter of target genes. There are several repressors of AR activity, most notable of which are calreticulin, cyclins, HBO1, Smad3, and TSG101, and p53 are all nuclear proteins. Cytoplasmic AR in absence of ligand, is tethered to the C-terminal end of FLNa through its hinge domain and LBD. Full length FLNa, cleaved at calpain cleavage site between repeats 15 and 16, releases FLNa (16–24), which colocalizes liganded AR to the nucleus. In the nucleus, FLNa disrupts the interaction between the N and C termini of AR and interferes with the binding of the co activator TIF2, causing repression of AR-transactivation. Mutation or deletion of hinge region disrupts AR–FLNa interaction, causing AR to bind to TIF2 and facilitates AR-mediated transactivation. Several mutations in the hinge region have been implicated in the androgen-driven prostate cancer.

SRY or SOX may interact with ARDBD, and as a result AR sequesters these tissue specific proteins, causing repression of PSME. Which could partially explain AR-mediated repression of PSME. Furthermore PSME also has eight AP1 at the repeat region. NFAT proteins by binding with AP3 interacts with AP1 binding proteins for optimal activity and AR negatively regulates PSME activity by interacting with AP1 protein as well. Therefore in the situation of Ca^{2+} ion influx, activates the function Ca^{2+} requiring proteins, such as calpain, calreticulin, causing repression of AR and activation of PSME, or calcineurin dephosphorylate NFATc1, which activates the transcription of PSMA (Fig. 5). This is the possible explanation for the androgen mediated suppression of PSME. Therefore tissue specificity and up-regulation in absence of androgen make PSME the best candidate for expressing toxic genes in the prostate in an androgen dependent environment.

ROLE OF FOLATE HYDROLASE IN PROSTATE CANCER

Folic acid is integral to the various metabolic processes within the body involving one carbon transfers in DNA synthesis, DNA methylation, formation of methionine which when decarboxylated is used in polyamine synthesis [Eto and Krumdieck, 1986; Jennings, 1995]. Our initial thinking was that PSM' the alternaive spliced form of PSMA may put the prostate at risk of folate deficiency because as a folate hydrolase it would allow for deglutamation of the poly-γ-glutamated folates which are the intracellular storage form of folates. Given that PSM' is less glycosylated being an intracellular protein, we now consider it likely that PSM' having folate hydrolase activity is unlikely. We

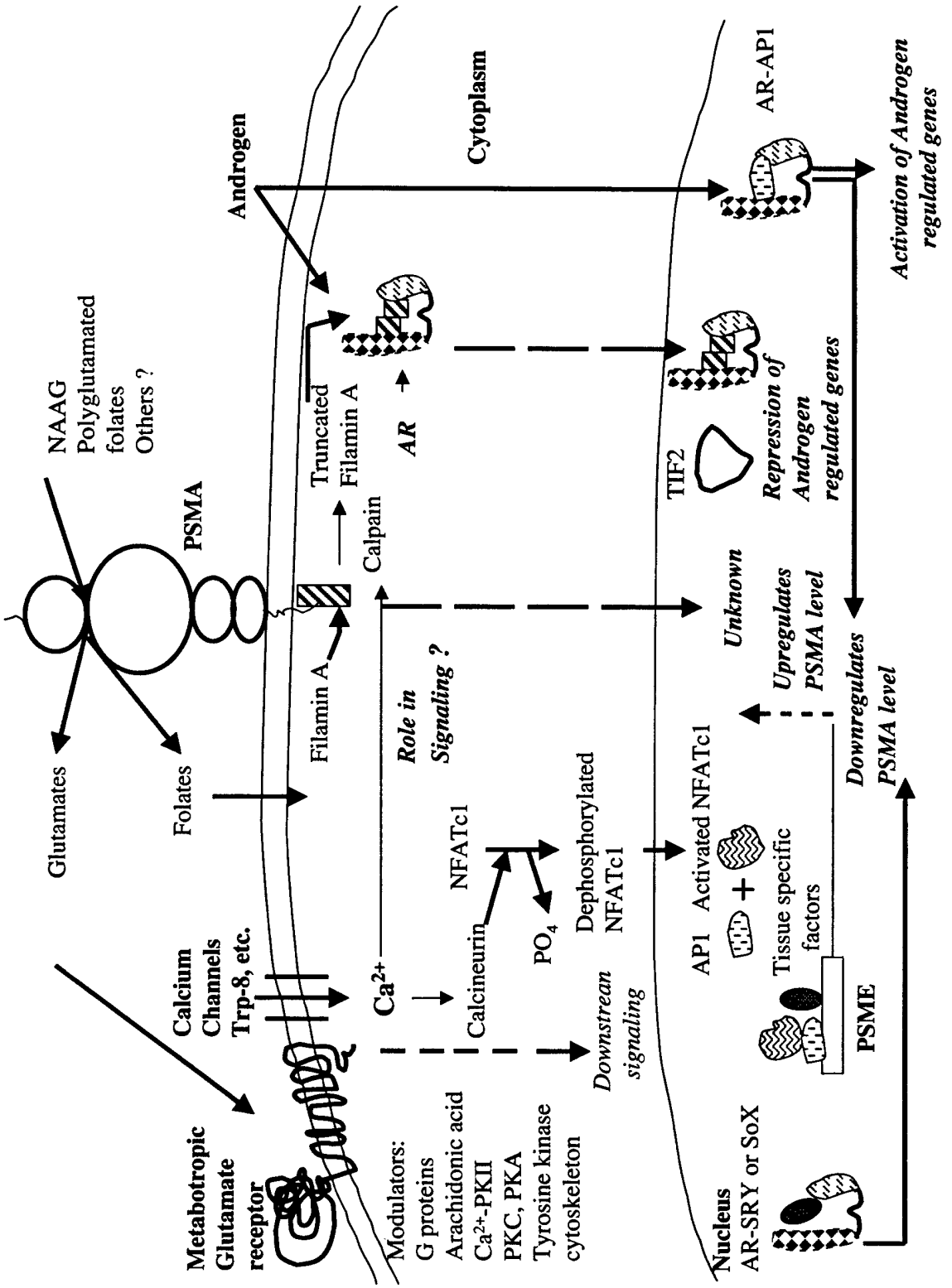
NAAG
Polyglutamated
folates
Others ?
PSMA
Glutamates
Folates
Androgen
Cytoplasm
Truncated
Filamin A
Calpain
AR
Filamin A
Role in
Signaling ?
TIF2
AR-AP1
Repression of
Androgen
regulated genes
Activation of Androgen
regulated genes
Calcium
Channels
Trp-8, etc.
Ca2+
NFATc1
Calcineurin
PO4
Dephosphorylated
NFATc1
Unknown
Upregulates
PSMA level
Downregulates
PSMA level
Metabotropic
Glutamate
receptor
Modulators:
G proteins
Arachidonic acid
Ca2+-PKII
PKC, PKA
Tyrosine kinase
cytoskeleton
Downstrean
signaling
AP1
Activated NFATc1
Tissue specific
factors
PSME
Nucleus
AR-SRY or SoX

Fig. 5.

at present have no idea what the intracellular form PSM' may be doing inside the cell. Outside the cell PSMA does bind and hydrolyze poly-γ-glutamated folate. But serum contains folate in a form that is not poly-γ-glutamated and is ready for transport into tissues. In the normal prostate, PSMA is at the apical surface, it is possible that it is exposed to a glutamated form of folate at this surface in the prostate cells. In cancers, it is not uncommon to have dead and dying cells in the tumor. As these cells would release their stored folate as poly-γ-glutamated folate, it is possible that PSMA enables cells to capture this folate by removing the γ-linked glutamates, thus freeing folate which then can be taken into the cell by folate binding proteins (FBP) or folate carrier systems. Given the interstitial pressure inside tumors because they lack lymphatics, and the cell death in hypoxic regions, that may provide a rational for the expression of PSMA on the tumor neovasculature, where it may be involved in helping capture the poly-γ-glutamated folates being released from dead and dying cells. Also because PSMA does internalize it may be that it has some transport property for poly-γ-glutamated folates that is as yet not understood.

SUMMARY

PSMA represents an excellent ideal cell surface protein for targeted therapy of prostate cancer and vasculotoxic therapy of non-prostate solid cancers. Clinical trials using antibodies that target the external domain of PSMA with imaging or toxic agents have been encouraging. PSMA is highly expressed in prostate cancer and is found to be strongly up-regulated in prostate cancer and it appears that calcium may be involved in the signaling for this increased expression. PSMA has to be glycosylated and to be in a dimer state to be enzymatically active. PSMA has activity as a carboxypeptidase with the preferred substrates NAAG and poly-γ-glutamated folate releasing glutamate upon hydrolysis of the substrate. The released glutamate may have a role in signaling. PSMA is internalized and exhibits a unique internalization motif, MXXXL. PSMA also binds to FLNa. FLNa is also associated with transducing extracellular stress to internal signaling, and PSMA may modify that signaling process.

ACKNOWLEDGMENTS

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Fig. 5. Cartoon showing the regulation of PSMA in prostate cancer cells. Negative regulation by androgen receptor (AR) and positive regulation Ca^{2+} is shown here. Polyglutamated folates get enzymatically cleaved to deglutamated folates and glutamates. The folates can enter the cells through reduced folate carrier (RFC) or folate binding proteins (FBP). The glutamates produced by the PSMA expressing cells can activate metabotrophic glutamate receptors which can get activated and can alter resting membrane potential, which causes efflux of Cl^- ions and influx of Ca^{2+} ions to compensate for the damage of the cells. Ca^{2+} ions can modulate the expression level of PSMA in many ways. Increased Ca^{2+} concentration, can activate inactive transcription factor NFATc1 (which is a transcriptional activator of PSMA enhancer), or cause activation of calpain, which cleaves FLNa. Truncated FLNa binds to AR and localizes to nucleus and suppresses AR-mediated transactivation. Normally AR would sequester AP1 or tissue specific transcription factors (e.g., SRY or SOX), causing inhibition of PSMA enhancer (PSME).

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The Prostate 58:200–210 (2004)

Comparative Analysis of Prostate-Specific Membrane Antigen (PSMA) Versus a Prostate-Specific Membrane Antigen-Like Gene

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BACKGROUND. Currently prostate-specific membrane antigen (PSMA) is showing promise both as an imaging and therapeutic target for occult prostate cancer metastases. First generation antibodies against PSMA are used for the FDA approved Prostascint™ monoclonal antibody scan and second generation antibodies are being developed for therapeutic targeting as well as imaging [1]. However, there have been reports describing PSMA expression in non-prostatic tissues including kidney, liver, and brain. As we had previously showed the existence of a human PSMA homolog, we set out to determine if this non-prostatic expression was due to expression of the PSMA or another gene.

MATERIALS AND METHODS. The PSMA homolog (PSMA-like) cDNA was cloned by screening a liver cDNA library. mRNA expression of the PSMA and PSMA-like genes was determined via Northern blot analysis using two different probes and protein expression confirmed in some tissues via Western blot analysis. Transcriptional regulation of the two genes was examined using reporter constructs driving luciferase expression.

RESULTS. The PSMA-like gene possesses 98% identity to the PSMA gene at the nucleotide level and is expressed in kidney and liver under the control of a different promoter to the PSMA gene. The PSMA gene is expressed in several human tissues and is most abundant in the nervous system and the prostate.

CONCLUSION. The non-prostatic expression of PSMA should be taken into consideration when designing clinical strategies targeting PSMA. *Prostate 58: 200–210, 2004.*

KEY WORDS: **cancer; tumor-vasculature; folate hydrolase; therapeutics; naaladase**

INTRODUCTION

Prostate-specific membrane antigen (PSMA), also known as folate hydrolase 1 (FOLH1) is an ideal potential target for use in determining patient management and therapeutic strategies against prostate cancer. It is highly expressed in both localized and metastatic prostate cancer [2–4]. Furthermore, PSMA is a type II membrane protein, with the majority of the protein located outside the cell readily available for therapeutic targeting or clinical imaging, or other diagnostic-type assays [5]. In addition, it now seems that therapeutic targeting of the PSMA molecule may

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have additional advantages; PSMA expression has been found in the endothelial cells of tumor vasculature of almost all types of tumors examined to date, including bladder, renal, breast, and lung carcinomas [4,6,7]. No PSMA expression has been reported in normal established vasculature. As such, a therapeutic approach targeted at PSMA could have broad implications for the treatment of many types of solid tumors. Accordingly, several groups are now attempting to utilize PSMA as a clinical and treatment target [1,8–11]. Clinical trials using radiolabeled antibodies to the external domain of PSMA have shown excellent results for imaging primary tumors and distant metastases not previously detected by conventional methods [10]. However, although PSMA is very highly expressed in normal and cancerous prostate, there are other tissues in the body that express low levels of PSMA or a similar mRNA including kidney, liver, and brain [11,12]. In order to use PSMA as a target, we wanted to know if this non-prostatic expression was in fact from the PSMA gene, and if it was not, what gene was expressed in these tissues. We recently mapped the PSMA gene to human chromosome 11p11.2, and a PSMA-like gene to chromosome 11q [13]. Both genes are the result of a genetic duplication that occurred 14 million years ago [13,14]. In order to determine where each of these two genes are expressed, we have cloned the PSMA-like gene and demonstrated methods to distinguish the two genes at the DNA, mRNA, and protein levels, which will aid in evaluating diagnostic and therapeutic strategies targeting PSMA.

MATERIALS AND METHODS

Fine Mapping of the PSMA-Like Gene

Primers that specifically amplify the PSMA-like gene were used to screen the Genebridge 4 Radiation hybrid panel (Research Genetics). The primers were: 5′-gccttcattttcagaacatctcatgcat-3′ and 5′-gtccatataaacttt-caagaatgtg-3′; the primer sequences were based on PCR sequencing/comparison of intronic regions of the PSMA-like gene by amplification of somatic cell hybrids as described below. Conditions were 35 cycles of 94°C 30″, 60°C 30″, and 72°C 1′. The results were analyzed using the server at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl.fordfay.

Genomic Sequence of the PSMA-Like Gene

Sixteen sets of primers were designed based on the genomic sequence of the PSMA gene (accession AF007544) as shown in Table I. Briefly, 100 ng of DNA from a somatic cell hybrid containing the distal portion of human chromosome 11q (NA11936 from Coriell Cell Repositories, Camden, NJ) was amplified for 35 cycles using 100 ng each of the primer pairs described in Table I. Conditions were: 95°C 30″, the annealing temperature indicated in Table I for 30″ followed by extension for 1′ at 72°C. In some cases as indicated, it was necessary to add DMSO to the reaction. Reactions were carried in a total volume of 50 μl out using 1 U of Expand High-fidelity Taq DNA polymerase in Buffer 2 supplied by the manufacturer (Roche, Indianapolis,

TABLE I. Primer Sequences and PCR Conditions Used to Amplify the PSMA-Like Gene

Exon	Bases	Sense primer	Sense primer sequence	Anti-sense primer	Anti-sense primer sequence	PCR product size expected
1	2488–2863	2529	tctctctctcgctcggattgg	2863	cgaagaggaagccgaggag	335 N/A
2	4994–5099	4341	tgtttctggccgcctatgcg	5254	agtatagtcctcctcagatg	914*
3	10726–10912	10630	caaagtacttttgtgtaactctgc	11082	cataggaaagtagttgacacgg	452#
4	18275–18376	18157	cctgaaggattcattcaccctc	18457	gacccttaattatcggctgaaca	300##
5–6	24400–25500	24323	atgtccaacagtccccatgcag	25593	gacatgcttagtccattgtacc	1270##
7	27927–28020	27871	gaaccgtttgaatgaaactgag	28058	ttacccaaatagccatccatgg	187*
8–9	35216–36281	35127	gcagatgctcaataagtgaatcc	36334	ccagcacataacagttacttgatc	1207#
10	37697–37816	37619	tagatgctattgagtcgtttgc	37867	aaactgagactcagataggctg	248#
11	39896–39978	39825	ctgggcttggtagtgtcctggg	40045	gcttggcaaacaagtcctggctac	220**
12	41911–41974	41792	tgtcgttaatatgggtcagctc	42035	ttaactagactgctgctcctag	243*
13	46402–46469	46317	tggtaggaatttagcagtggtc	46687	gatgctactaatgggctacctc	370**
14	53129–53220	53053	cttctggttaatggacatctag	53264	caatcccacactgaattcagtg	211◆
15	54364–54454	54278	agaatggggtttagtttaatgg	54536	tgagtcacttttggagtcag	258*
16–17	56661–57307	56614	ttgtaagctatccctataagag	57393	agttcagcaacagtcatgttag	779◆
18	62423–62515	62305	gggtggtcctgaaaccaatccc	62553	gtgatattacagaaaggagtc	248**
19	64209–64518	64127	atccaggaattgcagagtgctc	64586	ttcagttttaatccatagggag	459**

All sets of primers generated the indicated size product, except for exon 1 which was not able to be amplified. Annealing temperatures are indicated: *55°C, **57°C, #58°C, and ##60°C. ◆, Indicates an annealing temperature of 55°C in the presence of 5% DMSO.

IN), 0.2 mM of each dNTP, and 150 ng of each primer. PCR products were purified using the Wizard PCR Purification kit (Promega, Madison, WI) and directly sequenced on an ABI prism 3100 Genetic Analyzer. DNA from a somatic cell hybrid containing human chromosome 11p (NA11944) was used as a positive control.

RT-PCR

RNA was either made from cell lines using Trizol (Invitrogen, Carlsbad, CA), or obtained from Clontech (BD Biosciences, Palo Alto, CA). The bone marrow endothelial cell line (BMEC) was a kind gift from Dr. Malcolm Moore, Sloan-Kettering Institute for Cancer Research, NY, NY). RT-PCR was carried out using the Superscript Preamplification Kit (Invitrogen) according to the manufacturer's instructions. PCR to differentiate the PSMA and PSMA-like genes was carried out using 2 μl of the cDNA reaction as template. The primers used were: 5′-acagatatgtcattctgggaggtc-3′ and 5′-actgtgatacagtggatagccgct-3′. Initial denaturation was for 4 min at 95°C followed by 35 cycles of 95°C 30″, 60°C 30″, and 72°C 1′. Ten microliter of PCR product was digested with 10 U of EcoRI (New England Biolabs, Beverly, MA) in a total volume of 15 μl at 37°C for 2 hr. Products were resolved on a 1.5% agarose gel.

Cloning of the PSMA-Like cDNA

A liver cDNA plasmid (bacterial) library (Invitrogen) was screened as previously described [15] using a probe generated via PCR using the following primers: 5′-gtttataaaatcctccaatgaagc-3′ and 5′-gagcttctgtgcatcatagta-3′ (exons 2–7 of PSMA) or we used a probe spanning exons 10–16, generated by the same primers as used for the RT-PCR described above. Three prime RACE was carried out using the 3′ RACE system (Life Technologies, Gaithersburg, MD), with the primer 5′-ttgaggtgttcttccaacgac-3′ and a PSMA-like specific primer 5′-gacaaaagcaacccaatattg-3′. The cDNA sequence has been deposited in GenBank under accession number AF261715.

Northern Blot Analyses

Multiple Tissue Northern blots were obtained from Clontech. Hybridization with the hPSM-350 riboprobe [16] was carried out overnight at 56°C, followed by washing for 1 hr in 0.1× SSC and 0.1% SDS at 65°C as previously described [15]. Exposure was carried out for 5 hr at −80°C. The PSMA-like (Not1/Sal1 digest of the original clone in pSPORT) and β-actin probes were prepared using random-hexamer labeling (Invitrogen). Hybridization with the PSMA-like probe or the β-actin probe (Clontech) was carried out overnight at 42°C in 50% formamide (Hybrisol I, Intergen, Purchase NY), followed by washing at 42°C for 15 min in 0.2× SSC and 0.1% SDS. Quantitation of the relative amounts of PSMA expression in various tissues was carried out using the Image-J program with the gel analyzer plugin available from the NIH website http://rsb.info.nih.gov/ij/.

Regulation of the PSMA-Like Gene

The region of the PSMA-like gene that corresponds to the PSMA enhancer was cloned and sequenced using PCR with the following primers that incorporate artificial BamHI restriction sites (underlined) to amplify NA11936 DNA: 5′-cgcggatccgccttctaaaatgagttggg-3′ and 5′-cgcggatccggctactacataagtataagtc-3′ which produces a product of 1,648 bp. The PCR product was cloned into the BamHI site of the pGL3-B-PSM luciferase reporter vector containing the PSMA promoter and activity of the enhancer determined as we have previously described [17], with the addition that MDA PCa2b cells (ATCC, Rockville, MD) were maintained BRFF-HPCI Catalog no. SF-30 (Biological Research Faculty Facility, Ijamsville, MD) supplemented with 15% fetal calf serum (Invitrogen). The sequence of the PSMA-like enhancer region has been deposited in GenBank, accession number AF480875.

Immunoblotting

Western blotting for PSMA was carried out by transferring 25 μg of protein to PVDF membrane as described elsewhere [15]. The blot was probed with the Cyt-351 antibody (a gift from Cytogen corporation, Princeton, NJ) or PM2M-440 (a gift from Hybritech Incorporated, a wholly owned subsidiary of Beckman Coulter, Inc., San Diego, CA) and exposed using the ECL system (Amersham Biosciences, Piscataway, NJ). Other antibodies used in studies described here are J591 (a kind gift from Dr. Neil Bander, Weill Medical College of Cornell University, NY, NY) or PEQ-226 and PM1T-485 (kindly supplied by Hybritech Incorporated).

RESULTS

Fine Mapping of the PSMA-Like Gene

PSMA-like specific primers were designed based on PCR amplification and sequencing of a region of the PSMA-like gene that is homologous to part of the first intron of the PSMA gene. These primers were then used to screen the Genebridge 4 radiation hybrid panel, placing the PSMA-like gene 8.99 cR from CHLC.GATA45H10 and 0.40 cR from WI-6090 (LOD > 3).

Genomic and cDNA Sequence of the PSMA-Like Gene

We were unable to screen a cDNA library to obtain the PSMA-like cDNA clone, as we were unsure which tissue(s) it was expressed in, or indeed if it was expressed. Subsequently, we designed 16 sets of primers to the gene, the sequence of each set based on the intronic regions flanking the exons of the homologous PSMA gene. Using DNA from somatic cell hybrids retaining human chromosome 11q, we were able to generate products comprising each exon of the gene using the primers described in Table I, except for exon 1. All exons sequenced conformed to the GT-AG intron–exon boundary rule. The differences that we were able to determine at the genomic level are summarized in Table II.

In exon 12, a g → t change alters an EcoRI restriction enzyme site that is present in the PSMA gene so that it is no longer cleavable in the PSMA-like gene. To confirm this finding, we amplified exon 12 from the DNA of 18 unrelated people and somatic cell hybrid DNA containing either 11p or 11q. All individuals exhibited three bands after digestion, indicating the presence of one non-cleavable and one cleavable gene, confirming the g → t difference was not a polymorphism in the 11q

TABLE II. Nucleotide and Inferred Amino Acid Sequence Changes Relative to the PSMA Gene Deduced From Genomic Sequencing of the PSMA-Like Gene

Exon no. in PSMA	Nucleotide changes PSMA ⇒ PSMA-like	Amino-acid change PSMA ⇒ PSMA-like
1	Not present	N/A
2	No change	No change
3	nt 630 t → a	Threonine → threonine
	nt 584 t → c	Valine → alanine
	nt 594 a → t	Alanine → alanine
4	nt 739 c → t	Proline → serine
5	nt 777 c → t	Glycine → glycine
	nt 787 t → c	Tyrosine → histidine
	nt 877 g → a	Glycine → arginine
6	nt 948 c → a	Serine → serine
	nt 993 t → c	Aspartic acid → aspartic acid
	nt 1023 g → t	Glutamine → histidine
7	nt 1092 t → c	Tyrosine → tyrosine
	nt 1103 g → a	Arginine → glutamine
	nt 1150 a → g	Isoleucine → valine
8	nt 1237 c → t	Proline → serine
9	nt 1320 a → g	Threonine → threonine
10	nt 1454 t → c	Isoleucine → threonine
11	No changes	No changes
12	nt 1572 g → t	Glutamic acid → aspartic acid
13	nt 1665 g → a	Proline → proline
	nt 1684 c → t	Histidine → tyrosine
14	No changes	No changes
15	No changes	No changes
16	nt 2099 g → a	Serine → asparagine
	nt 2140 g → t	Valine → leucine
17	nt 2172 g → a	Lysine → lysine
	nt 2202 t → c	Serine → serine
18	nt 2239 g → t and nt 2241 a → g	Valine → leucine
	nt 2314 g → a	Arginine → arginine
19	nt 2442 a → t	Glutamic acid → aspartic acid
	nt 2459 a → c	Tyrosine → serine
	nt 2531 a → c	3′ UTR
	nt 2534 c → t	3′ UTR
	nt 2562 AG is deleted in PSMA-like	3′ UTR
	nt 2571 c → a	3′ UTR
	nt 2572 g → a	3′ UTR

gene (data not shown). To determine which tissues the PSMA-like gene might be expressed in, we carried out RT-PCR using primers spanning exons 10–16, followed by restriction enzyme digestion with EcoRI (Fig. 1).

Using this EcoRI non-cleavable site as a "sequence tag" for the PSMA-like gene, we were able to determine that liver and kidney showed a restriction enzyme banding pattern that corresponded to expression of both the PSMA and the PSMA-like genes, while the other positive tissues tested showed expression only of the PSMA gene. Next, we screened a 2.3×10^6 colony forming units from a liver cDNA library using a probe to exons 2–7 of the PSMA cDNA sequence. Subsequent clones were digested with EcoRI in order to exclude PSMA clones from further analysis. Only two PSMA-like clones could be identified this way although we found 26 full-length PSMA clones and two partial PSMA clones (beginning in exon 2 and 3, respectively). Both PSMA-like clones that we isolated began in a region corresponding to intron 5 and exon 6 of the PSMA gene. We next screened the library again, this time using a probe generated from the PCR product spanning exons 10–16 of the PSMA gene. This generated a further 12 PSMA-like clones (and no more PSMA clones), the most 5′ sequences of which corresponded to the same intron 5 of the PSMA gene. We confirmed the 3′ end of the gene using 3′ RACE and specific primers based on the PSMA-like gene sequence. The three longest clones from the library were sequenced, and the complete nucleotide and deduced amino acid sequence compared to that of the PSMA gene (Fig. 2). The sequence has been deposited in GenBank, accession AF261715. The longest open reading frame of the PSMA-like gene is homologous to the reading frame of PSMA. In addition, in vitro translation of the longest clone yielded the expected 46 kD protein (data not shown). At the mRNA level PSMA-like is 98% homologous to PSMA, and the protein shows 97% identity and 98% similarity to PSMA in the translated region. It should be noted, however, that the expected size of PSMA-like *in vivo* is 46 kD, while PSMA is 100–120 kD after glycosylation of its 84 kD core.

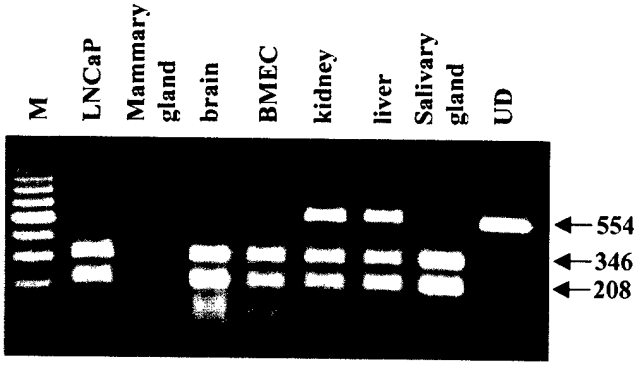

Fig. 1. Expression of the prostate-specific membrane antigen (PSMA) and PSMA-like Genes. RT-PCR followed by gene-specific restriction enzyme digestion was used to differentiate expression of the PSMA and PSMA-like genes in various tissues. The PSMA gene yields two bands of 346 and 208 bp following digestion with EcoRI, while the PSMA-like gene remains uncut at 554 bp. M = 100 bp ladder (Gibco); LNCaP, prostate cancer cell line; BMEC, bone marrow endothelial cells; UD, undigested.

Expression Pattern of the PSMA and PSMA-Like Genes

To determine what tissues express PSMA while avoiding detection of the PSMA-like gene, we used a probe from the first three exons of PSMA which are not found in the PSMA-like cDNA sequence (probe p350). Northern blot analysis confirmed expression of PSMA in the prostate, brain, kidney, small intestine, liver, and spleen (Fig. 3a,b). After prostate, the next five highest expressing regions were all from the brain, and the other tissues were all at levels less than 10% of that of PSMA in normal prostate (Table III). Similarly, Western blot analysis using the Cyt-351 and PM2M-440 antibodies showed protein expression in the hippocampus and amygdala compared to that seen in the prostate cancer cell line MDA PCa2b (Fig. 4a,b). The Cyt-351 antibody binds to the intracellular region of PSMA that is deleted from the PSMA-like gene [18], while PM2M-440 binds to a region within amino-acids 135 and 173 of PSMA (personal communication with Harry Rittenhouse, Hybritech Incorporated), which is not found in the PSMA-like cDNA.

PSMA mRNA expression was either negligible or not detected in thymus, testis, ovary, colon, leukocytes, heart, placenta, lung, muscle, and pancreas. In addition, we cloned and sequenced PSMA expressed in tumor neo-vasculature and confirmed that it was not PSMA-like. However, the clone sequenced did contain two changes at nt 1784 (G → A; Gly → Asp) and nt 1817 (A → G; Asn → Ser). To determine the relative expression of the PSMA-like gene, we next probed with its cDNA in entirety (Fig. 5a–d). This would detect both PSMA (2.7 kb) and PSMA-like (2.0 kb) mRNA. PSMA-like was expressed at similar levels to the PSMA gene in adult kidney and liver, but not in any other tissues including fetal kidney, liver, brain, and lung. Interestingly, PSMA is expressed in fetal liver and kidney, and also in adult trachea and spinal cord.

Regulation of the PSMA-Like Gene

We cloned a prostate-specific enhancer from the third intron of the PSMA gene [17,20]. As we had shown that the PSMA-like gene was not expressed in the prostate (Fig. 1 and data not shown), we were interested to compare the sequence of the two genes

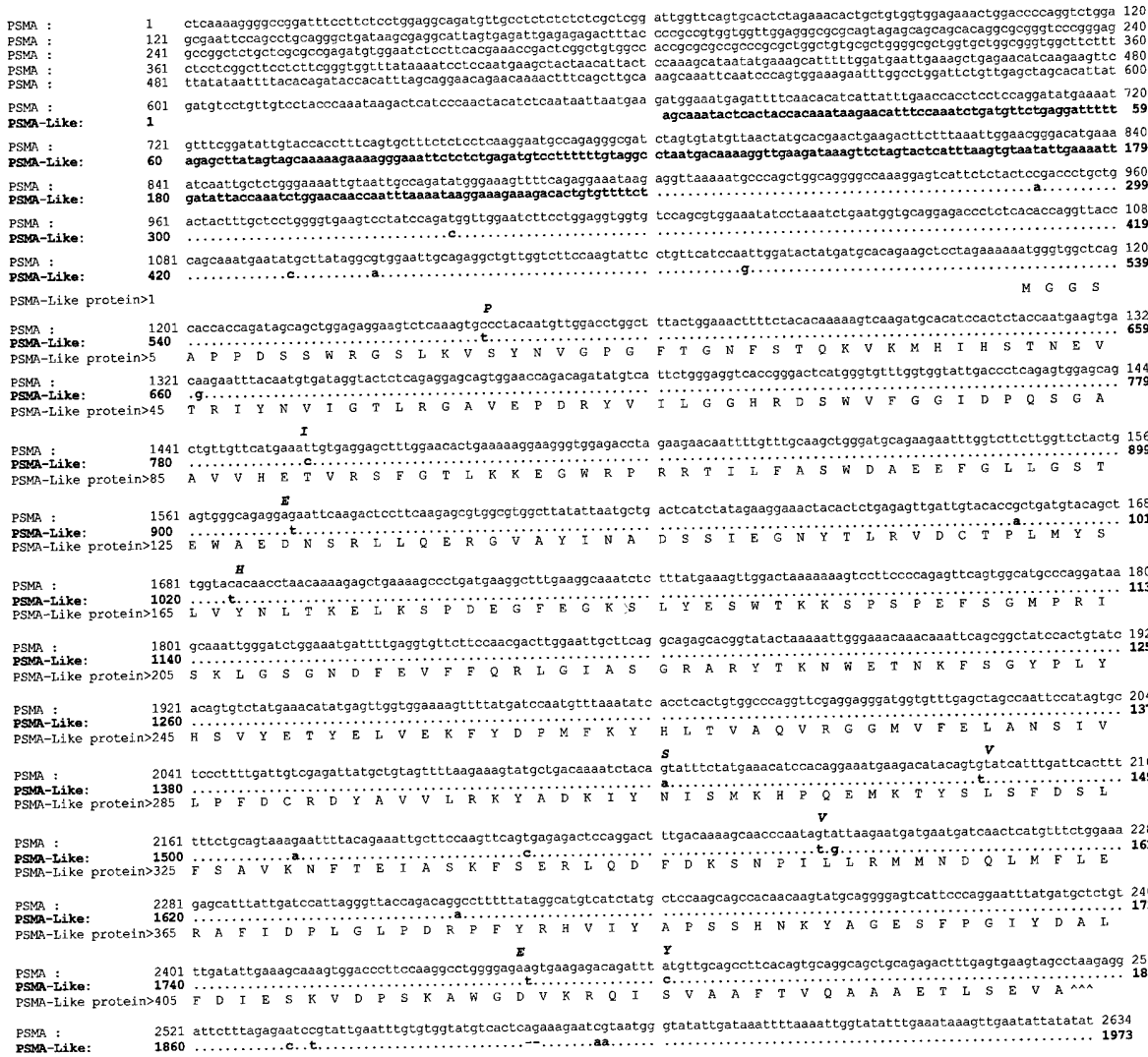

Fig. 2. Nucleotide and deduced amino acid sequence alignment of the PSMA-like gene compared to the PSMA gene. Differences in nucleotide sequence are indicated in bold lower-case lettering, while amino acids found in PSMA and not PSMA-like are indicated in bold capitals above the sequence. The GenBank accession numbers for PSMA-like and PSMA are AF261715 and M99487, respectively.

"enhancer" regions. PCR using primers homologous to the PSMA enhancer were used to amplify DNA for sequencing from the 11q-containing hybrid. Surprisingly, the sequence of the two intronic regions is 99.3% identical. Because the minor differences between the two regions might alter novel prostate-specific transcription factor binding sites, we tested the PSMA-like enhancer region for its ability to drive luciferase reporter gene expression in combination with the PSMA promoter (Fig. 6).

The PSMA-like enhancer was able to drive luciferase expression equally as well as the PSMA enhancer in C4-2 prostate cancer cells. In addition, like the PSMA enhancer, there was no activity in the breast cancer derived cell line MCF-7. The PSMA-like enhancer showed approximately equal activity in both the C4-2 and MDA PCa2b prostate cancer cell lines, however, the PSMA enhancer repeatedly showed a 2.5-fold increase in activity in the MDA PCa2b cell line over its expression in the C4-2 cell line.

As the enhancer of the PSMA-like gene was able to activate prostate-specific gene expression, we wanted to examine the region of the PSMA-like gene that corresponded to the PSMA promoter, but had been unable to amplify any region of it or exon 1. A BLAST comparison with the high throughput genomic sequence database revealed homology of the PSMA-like gene with a contig of 27 unordered pieces (GenBank accession AC024234). BLAST analysis of this contig against the promoter region and exon 1 and 2 of the PSMA gene in an attempt to identify a PSMA-like promoter showed a deletion in AC024234 in the region

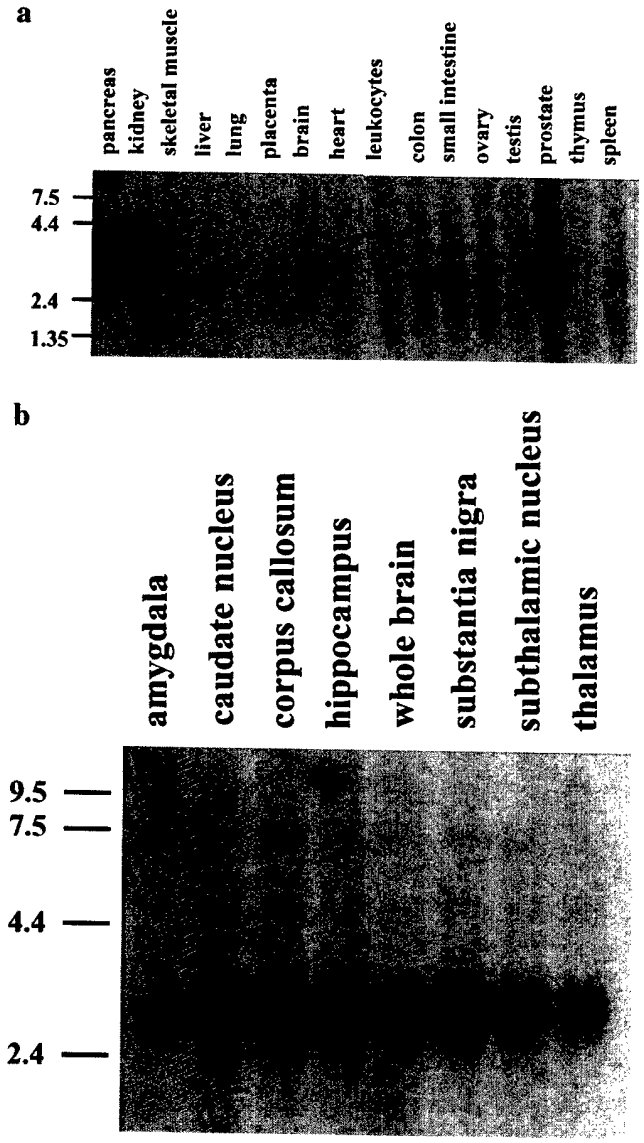

Fig. 3. Expression of PSMA as determined by Northern blot analysis using a probe that will not detect the PSMA-like gene. **a**: Expression of the 2.6 kb PSMA transcript is clearly strongest in the normal prostate, although expression of PSMA can also been seen in other tissues. **b**: Expression of PSMA is also found in the brain, although the levels vary depending on the region examined. RNA marker sizes are indicated in kb.

TABLE III. Relative Amounts of PSMA in Various Tissues

Tissue	Expression (relative to normal prostate)
Prostate	100
Corpus callosum	59
Substantia nigra	35
Caudate nucleus	20
Hippocampus	17
Subthalamic nucleus	12
Whole brain	9
Kidney	9
Thalamus	7
Small intestine	6
Liver	5
Amygdala	4
Spleen	2

The amount of PSMA in various tissues was determined using densitometry of the Northern blot analysis shown in Figure 5. Expression of PSMA in prostate was arbitarily set at 100. The next five highest expressing tissues are all from the brain, while the next highest expressing organ, kidney, has less than 10% of the amount of PSMA that is seen in the normal prostate. All values were adjusted for β-actin expression (data not shown).

corresponding to approximately 500 nt upstream of the PSMA transcription start site, the entire first exon, and the first 371 nt of intron 1. To confirm this result, we designed primers to either side of the deletion, and used them to amplify DNA from five unrelated people. As the primers could amplify both PSMA and PSMA-like, we expected to see approximately a 330 bp product generated from all the DNA samples if the deletion had occurred, and a 1.5 kb band from the PSMA gene. All five DNA samples produced a 330 bp band, while the 11p hybrid produced a 1.5 kb band (from the PSMA gene) and the 11q hybrid a band of approximately 330 bp, verifying that deletion of the region corresponding to the promoter and exon 1 of PSMA had been deleted in the PSMA-like gene (Fig. 7). No 1.5 kb band from the PSMA gene is visible in the human DNA lanes, probably due to preferential amplification of the smaller product.

To see if there is a promoter close to the transcription start site of the PSMA-like gene, we analyzed 550 nt of sequence upstream using the promoter prediction program at http://www.fruitfly.org/seq_tools/promoter.html, which uses a neural networking method to predict promoter regions. A promoter was predicted 150 nt upstream of the 5′ end of the longest clone obtained. In addition, there is a CCAAT box 161 nt and a TATA box 28 nt from the transcription start site.

DISCUSSION

In order to resolve the origin of the so called "non-prostatic" expression of PSMA, we cloned the PSMA-like gene and determined its expression pattern. The PSMA and PSMA-like genes arose from a duplication event of the original gene approximately 14 million years ago [14,20]. The site of the original gene was most likely 11q14.3, as this region has conserved synteny with the location of the single murine PSMA homolog, folh1 at 7D1-2 [21]. The finding that the PSMA-like gene maps to the schizophrenia disorder type II locus is

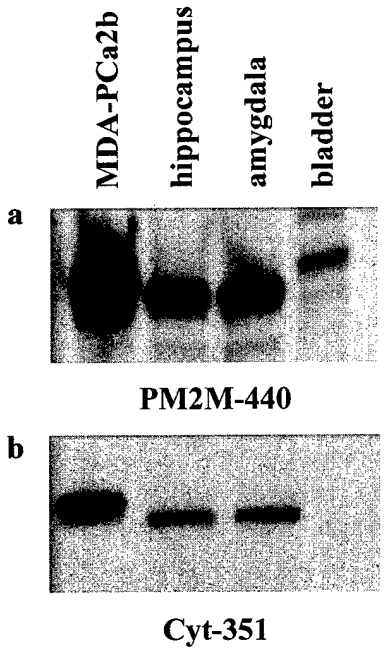

Fig. 4. PSMA protein expression via immunoblot analysis. **a**: Using antibody PM2M-440 and (**b**) antibody Cyt-351. Both antibodies are specific for PSMA because they bind to regions of the protein that is missing in the PSMA-like protein. Densitometry of this blot reveals approximately 40-fold less PSMA protein in the brain tissues amygdala and hippocampus than is seen in the prostate-cancer cell line MDA-PCa2b. The size of the proteins is around 130 kD, although it seems that the brain might glycosidate PSMA differently to the prostate. A slightly larger band appears only with the PM2M-440 antibody in the bladder sample; we assume this is non-specific as it is not detected with the Cyt-351 antibody and Northern blot analysis showed no expression of PSMA RNA in the bladder.

particularly interesting as a disruption in the NAALADase activity of PSMA has been implicated in the pathogenesis of schizophrenia [22].

Non-Prostatic Expression of the PSMA Gene

The aim of this study was to analyze the possible expression of PSMA in non-prostatic tissues. We have shown here that PSMA is in fact expressed in a number of non-prostatic tissues. This expression is not due to other hypothetical or known homologs of PSMA as described in the EMBL database, as our p350 probe would either not detect these mRNAs, or would bind to an mRNA of a significantly different size to PSMA [23,24] and observations from EMBL database (http://www.ensembl.org/Homo_sapiens). In addition, it should be noted that although there are two "homologs" of PSMA shown on chromosome 2 in the EMBL database, we have tested the possible existence of these using PCR of somatic cell hybrids containing human chromosome 2, and concluded that they are the result of artifact and in fact do not exist (data not shown).

Regulation of Expression of the PSMA and PSMA-Like Genes

Presumably the loss of the genomic DNA segment in the PSMA-like gene that corresponds to the promoter and exon 1 of PSMA occurred subsequent to the duplication event. The similarity between the PSMA and PSMA-like genes is remarkable, yet the very few differences between them allows us to learn more about the biology of PSMA. Despite the fact that the "enhancer" regions of the two genes are 99.3% identical, the PSMA enhancer is more than twice as active than the PSMA-like enhancer combined with the PSMA promoter in MDA PCa2b cells. However, both enhancers work equally well in C4-2 cells. This suggests that some of the few sequence differences correspond to important enhancer factor binding sites for factors that are present only in MDA PCa2b cells. It has previously been reported that a 330 bp core region contributes most of the activity of the PSMA enhancer [19]. Comparison of the two enhancer sequences reveals that two of the differences abolish binding sites for ATF/CREB and CEBP although there could be other sites as yet unrecognized that are also altered. Dissection of the factors controlling PSMA expression is important as we and others have been utilizing the PSMA enhancer to develop gene therapy strategies [17,25].

As the PSMA-like enhancer is capable of activity when combined with the PSMA promoter, we might expect to see prostatic activation of the native PSMA-like promoter via the PSMA-like enhancer. However, this clearly does not occur as we do not see expression of the PSMA-like gene in the prostate. One possible explanation for this is that an insulator region exists between the PSMA-like enhancer and promoter, blocking expression of the PSMA-like gene in prostate-derived tissue [26]. Similarly, although the region corresponding to the PSMA-like promoter in the PSMA gene is almost identical, it is not activated by the PSMA enhancer or we would expect to see a 2.0 kb mRNA band in prostate tissue via Northern blot analysis. This supports the idea of an insulator region because previously the PSMA enhancer has been shown to be capable of activating even the most minimal promoters such as the TK gene promoter [19].

Clinical Targeting of PSMA

Exon 1 of the PSMA gene encodes for the single transmembrane domain, however, this region is lost from the PSMA-like gene. As the PSMA-like cDNA sequence lacks a transmembrane domain, we expect

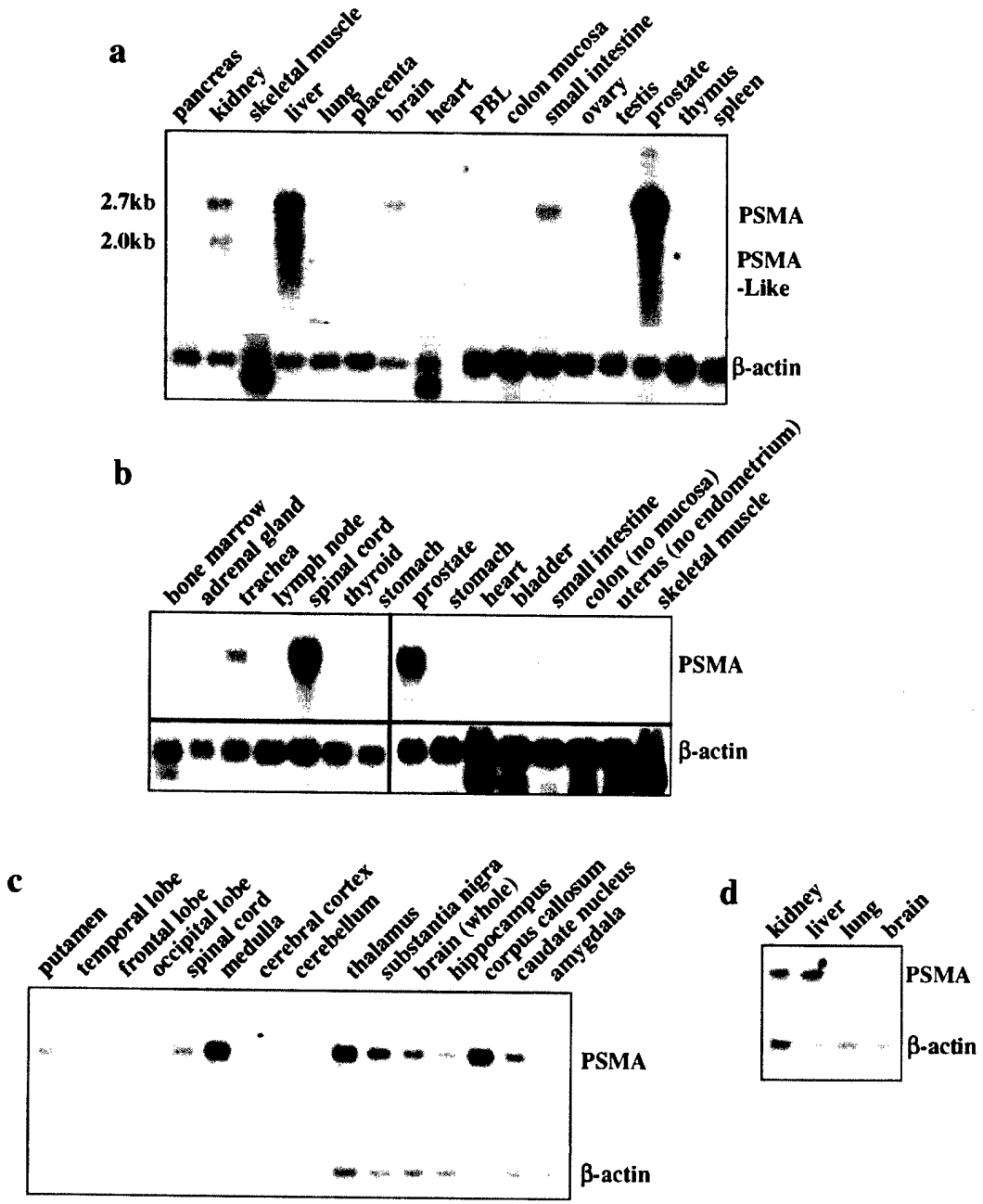

Fig. 5. PSMA and PSMA-like mRNA expression in various tissues as seen via Northern blot analysis. Expression of PSMA vs. PSMA-like was determined by utilizing a probe that detects a 2.7 kb band corresponding to PSMA, and a 2.0 kb band corresponding to PSMA-like. **a** and **b**: PSMA and PSMA-like expression in normal human tissues, (**c**) PSMA expression in brain tissues, and (**d**) PSMA expression in fetal kidney and liver. β-Actin was used as a loading control.

the PSMA-like protein to be located within the cytosol, and, therefore, not subject to the glycosylation that is undergone by the PSMA protein [27]. Barinka et al. [28] and our own results [29] have demonstrated that glycosylation of PSMA is indispensable for enzymatic activity. Based on these findings, we would predict that expression of the PSMA-like protein would not result in enzymatic activity that could affect prodrug therapies targeted at PSMA. In addition, due to the hypothesized intracellular localization of the PSMA-like protein, cells expressing it would not be targeted by antibodies or other extracellular agents directed against PSMA.

Even though our studies use normal prostate RNA for comparison with other normal tissues, it should be remembered that prostate cancer is reported to contain a 10-fold increase in PSMA mRNA expression. Nevertheless, the expression of PSMA mRNA in the various

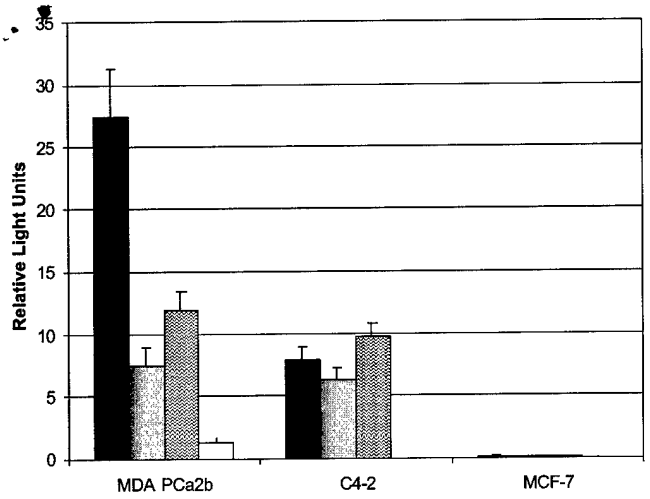

Fig. 6. Comparison of the PSMA and PSMA-like "enhancer" regions. Potential of the PSMA-like enhancer to drive a luciferase reporter gene in combination with the PSMA promoter is reported in relative light units after adjusting for transfection efficiency. The PSMA-like sequence can operate as a tissue-specific enhancer, as evidenced by reporter gene expression in the prostate cancer cell lines MDA PCa2b and C4-2, but not the breast cancer line MCF-7. Interestingly the enhancer has the same activity as the PSMA enhancer in C4-2 cells, while in MDA PCa2b the PSMA enhancer induces more than twice as much reporter gene expression than the PSMA-like enhancer clones. The black bar indicates the PSMA promoter/enhancer construct, the wavy bars are two individual clones of the PSMA promoter/PSMA-like enhancer construct, while the white bar is the PSMA promoter alone. The standard deviation of triplicate experiments is shown.

regions of the brain, the spinal cord, liver, and kidney is of significant concern and needs to be addressed when designing therapeutic strategies utilizing PSMA as a target. However, the brain and spinal cord are protected by the blood–brain barrier. In addition, there is no immunohistological evidence of PSMA protein expression in the brain or liver, despite several studies of these tissues (reviewed in Tasch et al. [30]). The same studies have shown positive staining for PSMA in the kidney, where it is expressed weakly in a subset of proximal tubule cells. It is possible that PSMA protein expression is regulated post-transcriptionally, so that the amount of mRNA present is not an indicator of actual protein. Prostate cancers contain over 1,000-fold greater levels of PSMA protein than found in liver or brain as determined quantitatively by RIA [12]. Most importantly extensive imaging studies [31] and phase one trials using cytotoxic radiolabeled, humanized antibodies against PSMA [32] have shown specificity for prostate, prostate cancer, and the neovasculature of other solid tumors including renal cell carcinoma. With both treatments there was a low frequency of side-effects, and in addition it seems the treatment with the antibody conjugated to β-emitters might be in the therapeutic range, as some of the patients with prostate cancer had as much as an 85% reduction in PSA. Consistent with a blood–brain barrier effect, immuno-targeted antibody approaches with radiolabeled antibodies against PSMA used for imaging do not show localization in the brain [33]. It is also heartening that there have been no reports of tissue injury in phase II trials stimulating the immune system against PSMA. Indeed, the trials have shown little if any toxicity, but a positive response rate in 30% of the patients [34].

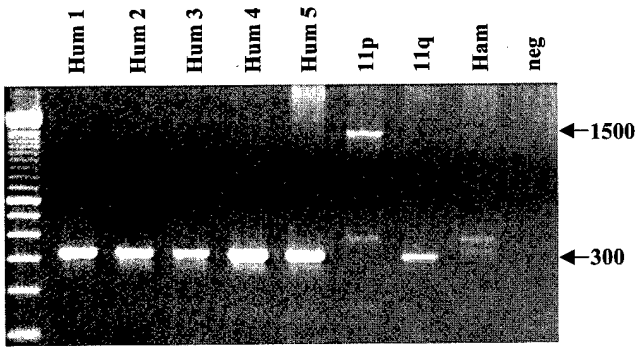

Fig. 7. Deletion of the original PSMA-like promoter, exon I and part of intron I. PCR analysis was performed using primers based outside the region predicted to be deleted in the PSMA-like gene. Amplification of the predicted 300 bp band if a deletion had in fact occurred is seen in all five human DNAs (hum I–5) and DNA from the IIq human-hamster hybrid (IIq). Amplification of the 1,500 bp band in the homologous region of the PSMA gene is generated from the IIp hybrid (IIp). "Ham" indicates the parental hamster DNA of the hybrids.

CONCLUSIONS

We have cloned and characterized a gene that is highly homologous to PSMA, and determined ways to distinguish the two genes at the DNA, mRNA, and protein levels. PSMA can be a useful clinical target for prostate cancer, however, both the presence of the PSMA-like gene and expression of PSMA in other tissues should be taken into consideration when designing diagnostic and therapeutic strategies. In addition, the small but significant differences between the evolutionary twins, PSMA and PSMA-like, allows us to learn more about the function and regulation of PSMA.

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